Cotton and Microorganisms

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Abstract

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Many microorganisms are found on cotton. When cotton is processed, dust and microorganisms and their associated products (such as endotoxins) become airborne. The airborne dust, microorganisms, and endotoxins generated cause some workers in mills to become ill. The main illness is a respiratory disease called byssinosis. Over the last 20 yr, significant research has gone into studies of byssinosis and mechanisms thought to be important in its etiology. The chapters of this book are written by researchers who have devoted considerable time to these studies. The microorganisms associated with byssinosis are identified by the authors. Each chapter summarizes the results of studies done by the author and others who have done related work.

Keywords: microorganisms, byssinosis, cotton dust, endotoxin, gram-negative bacteria, gram-positive bacteria, fungi, respiratory disease, cotton mills, carding, cotton cultivars, harvesting, storage, bracts, Classer's samples, fiber quality, textile mills

While supplies last, single copies of this publication may be obtained at no cost from Preston E. Sasser, Cotton Incorporated, 4505 Creedmoor Rd., Raleigh, NC 27612.

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Contents

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Introduction

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Cotton, *Gossypium* spp., is one of the world's most valuable fibers. Fibers of commercial value are also produced by flax, hemp, agave, and jute plants. Sisal is a hemplike fiber taken from agave plants. Hemp actually refers to various fibrous shrubs. When these fibers are processed, pulmonary problems are unlikely to occur in workers handling "hard" fibers (jute and sisal) but are likely in workers handling "soft" fibers (soft hemp, cotton, and flax).

Cotton in the boll is initially sterile, but within 48 hr of boll opening the cotton fiber acquires a large flora of microorganisms that persists at high levels throughout the fruiting season. Different genetic varieties of cotton have been studied to search for microorganism-resistant strains, but microorganisms seem to occur about equally on all varieties. Cotton, flax, hemp, and jute contain large numbers of bacteria, especially gram-positive *Bacillus* and *Corynebacterium* species and also gram-negative microorganisms.

Cotton (a form of cellulose) is harvested and processed into yarn without being subjected to treatments that would remove the microorganisms associated with the fiber and plant parts (mainly leaves and bracts) that accompany the fiber into the early stages of processing. Although all of the plant parts carry large numbers of microorganisms, it has been estimated that 67 percent of the microorganisms and 89 percent of the endotoxin (a component of the cell wall of GNB that is believed to cause respiratory problems in mill workers) are associated with the cotton lint itself. Neither prolonged heating nor washing is used early in cotton processing; instead the cotton is subjected to mechanical ginning for seed removal and drying. Other crops also carry resident microorganisms into the processing stream, but microorganisms from these crops are often eliminated or reduced during processing.

Once ginned, cotton is shipped to mills in bales, and the bales are then opened and carded, releasing large numbers of microorganisms, fragments of microorganisms, and plant parts into the air. Some of the gram-negative bacteria (GNB) may die, but the spores (the resistant form) of the gram-positive bacteria can persist for years. When the microorganisms and dust become airborne, they put mill workers at risk for developing respiratory problems.

Many factors are involved in creating the conditions that lead to respiratory problems in mill workers. Current Federal laws specify a maximum allowable dust exposure for mill workers but do not restrict exposure to microorganisms. Most processing mills were constructed many years ago without adequate consideration to ventilation. The majority are located in the southern part of the United States where high temperatures encourage multiplication of bacteria. The advent of mechanical harvesting of cotton has also increased the amount of plant parts contained in the cotton when it reaches the gin and the mill, and these plant parts are also contaminated with microorganisms.

Once the airborne particles and microorganisms are inhaled, the lungs depend upon coughing and phagocytosis, a cellular mechanism, to remove the foreign components. But coughing removes only some of the particles (usually the larger), and phagocytosis is not an efficient method for eliminating particles.

The microorganisms usually found on cotton are rarely human pathogens, and some do not grow well at the temperature of the human body. There is no evidence that respiratory problems of people working in cotton mills result from infections of the human lung. The bacteria from cotton dust do not multiply in the body—rather the respiratory problems result from some fragment of the bacteria stimulating human cells to produce cytokines. Cytokines trigger the production of inflammatory mediators which then produce an inflammatory response in humans.

Repeated exposure to dust and airborne contaminants in the mill can lead to a disease called byssinosis in mill workers. Endotoxin is believed to be the cause of the acute syndrome described as "mill fever" and may play an important role in causing the chronic pulmonary disease byssinosis. Byssinosis is a problem in the early stages of cotton processing, especially when low-grade cotton is processed.

Although the incidence of byssinosis is on the decline (thanks to engineering changes in mills), the disease is still prevalent in mills today. Understanding the role of microorganisms and their relationship to cotton could lead to procedures to remove microorganisms from cotton in the field, gin, or mill and thereby further decrease the occurrence of the disease. Conversely, microorganisms could be prevented from getting on the cotton fiber initially or they could be trapped on the cotton so that they cannot become airborne.

Mills would be safer workplaces if airborne levels of dust and microorganisms could be reduced, and many research projects have concentrated on doing just that. Large-scale studies of cotton washing have been carried out on a wool scouring line, a batch processing line, and a continuous processing line. In each case washing reduced the counts of microorganisms and the level of endotoxin in the washed lints. Washing was most effective in the batch processing and continuous processing lines. The decline in pulmonary function associated with inhalation of cotton dust was markedly diminished when subjects were exposed to cotton washed on a continuous line system. Studies of the dust generated when washed and unwashed cottons were carded showed a significant reduction in the bacterial population and endotoxin content in dust generated from washed cotton. (Washed cotton, however, does not process easily in mills.) An oil spray on cotton before carding also reduced the number of airborne microorganisms. Cotton grown in greenhouses or harvested from the field by hand also has a lower number of bacteria, but these methods are not economical.

This book summarizes these and other research studies and provides future students with the knowledge and database upon which future research with cotton can be undertaken. The focus of the book is on studies that have explored the etiology of byssinosis and the role of microorganisms and endotoxins as the causal agent. The problems associated with byssinosis are also discussed. The findings presented also demonstrate that significant progress in solving a problem (in this case byssinosis) can be made by the collaboration of numerous investigators from various institutions.

More questions are raised than answered, but these questions should help inspire new approaches to these problems in future research. For example, in the future it may be possible to genetically engineer or isolate special strains of bacteria that produce little or no endotoxin. (A mutant strain of *Escherichia coli* that produces only small amounts of endotoxin has already been found.) These special bacteria could be inoculated onto cotton as the bolls open to prevent the usual microorganisms from populating the cotton fiber. Another approach would be to spray cotton in the field with chemicals that bind endotoxin to the cotton.

The results presented in this book from the studies on cotton can be applied to other environments and other crops. The findings should help investigators in different fields and demonstrate the importance of studying the microbiological flora of an agricultural crop.

Chapter 1. Why Study the Microbiology of Cotton?

Robert R. Jacobs

Public Health Basis for Studying the Microbiology of Cotton

The basis for studying microorganisms on cotton can be seen in the observation made in 1713 by Bernadino Ramazzini who said that "those who card flax and hemp so that it can be spun and given to the weavers to make the fabric find it very irksome. For a foul and poisonous dust flies out from these materials, enters the mouth, then the throat and lungs, makes the workman cough incessantly, and by degrees brings on asthmatic troubles" (Ramazzini 1964). This observation was the first to associate specific respiratory health effects with exposure to dust generated from the processing of vegetable fibers. While cotton was not specifically included in the list of fibers described by Ramazzini, subsequent reports describe similar health effects from cotton dust (Rooke 1976).

Respiratory Disease in Cotton Workers in the United Kingdom

Cotton occupies a unique place in the history of the industrial revolution because its use served as the basis for the development of the first mechanized industry, the textile industry, in the United Kingdom and the United States. Furthermore, it was this development of the textile industry that brought large numbers of workers into a single workplace and laid the foundation for conditions that contributed to increased disease morbidity and mortality among the working population.

The development of the modern cotton textile industry had its roots in the invention of the spinning jenny by Hargrave in 1767. This machine produced greater quantities of yarn more rapidly and was the first step in the transition of textiles from a cottage industry to a factory system. The increased yarn production prompted the development of the waterframe loom by Arkwright in 1771. The waterframe loom provided a means to use the abundant yarn generated by the spinning jenny. However, it required water for power and fostered the development of mills near a water supply. In 1779 Compten developed the mule jenny which again increased the rate of yarn production, and with the development of the steam engine by Isaac Watts in 1785, Cartwright's power loom was capable of handling the excess yarn production.

The development of steam power eliminated the need for a water supply, and mills began to move to cities for abundant and inexpensive labor. In 1773, Manchester—the center of the textile industry in the United Kingdom—had a population of 13,787 (Rooke 1976). By 1831 the population had increased to 270,363. This growth was paralleled by the demand for cotton, which increased from 5 million lb in 1778 to 274 million lb by 1831. The growth in production and centralization of the textile industry also resulted in the development of poor working conditions. A typical mill environment was described as follows: "Bear in mind the heat, the lack of movement of air, the complete lack of ventilation of the privies on each floor of the mill and then add to that the stench of hot, rancid animal fats that were used to oil the machinery and you will have some idea of the basic working environment, and this without taking into consideration that in some departments of the mill the dust was so thick that it was impossible to see across the room" (Rooke 1976).

Conditions such as these, coupled with the fact that much of the workforce consisted of young children and women, prompted the development of the first factory legislation in England. Initial health and safety legislation dealt not with respiratory disease in the textile industry but with the use of child labor. In 1802 the Health and Morals of Apprentices Act was the first state intervention in private enterprise (Luxon 1984). This act and those that followed pertained only to the textile industry. Only in 1864 were these acts expanded to include other industries.

The most frequently cited description of respiratory disease in cotton textile workers in the United Kingdom is that of Kay (1831). Kay observed that workers in the initial stages of processing and workers exposed to "coarse and therefore dirty cotton" had a higher prevalence of respiratory disease. Prior to Kay's description, a febrile response was associated with exposure to cotton in

1784 (Meiklejohn 1959), and the potential health effects from exposure to cotton dust were reported as early as 1785 (Murray 1959). The febrile response was later described in 1860 as "mill fever" and was associated with the first exposure to cotton dust (Rooke 1976). This report also described the reduction of symptoms that occurs over the work week.

The first term used to describe the chronic respiratory response to cotton dust was "lyssinosis" by Ludwig Hirt in 1871. In 1877 Proust questioned the use of the term lyssinosis, suggesting that "byssinosis" was the correct term. Lyssinosis was derived from the Greek word meaning rabies, whereas byssinosis is derived from the Latin word "byssus" meaning fine white fiber. The term byssinosis was later used by Sir Thomas Oliver in 1902 and has been used since that time to describe the "Monday [chest] tightness" found in those exposed to the dust of cotton, flax, jute, or hemp (Massoud 1964).

Respiratory Disease in Cotton Workers in the United States

The development of the textile industry in the United States paralleled that in the United Kingdom and was largely the result of technology brought to the United States by Samuel Slater, a former worker in a British textile mill. By 1815 the textile workforce in the United States numbered about 100,000 and was centered in New England (Teleky 1948). This development, while creating conditions in the workplace that resulted in increased morbidity and mortality, did not serve as the basis for the development of health and safety legislation in the United States as it did in the United Kingdom. In fact health and safety legislation in the United States was largely based on state initiatives until the twentieth century when the Federal Occupational Safety and Health Act (PL 91–596) was enacted in 1970. The first state initiatives in the nineteenth century focused on child labor, the employment of women, and the length of the workday and were not specifically based on problems unique to the textile industry. Furthermore these initiatives were inconsistent from state to state and had no legal authority across state boundaries. Because no uniform laws existed to protect the health and safety of workers in the textile industry, recognition of and response to respiratory disease in the cotton textile industry was delayed for over a century in the United States.

The widespread recognition of an association between exposure to cotton dust and respiratory disease did not occur in the United States until the 1940's. As late as 1945 the U.S. Department of Labor did not associate exposure to cotton dust with the development of respiratory disease, stating that byssinosis was not a problem because of efficient dust control in textile mills and because textile workers frequently changed jobs (U.S. Department of Labor 1945). However, reports of byssinosis were appearing in U.S. medical literature, including those by Bolen (1943), Bogan (1946), McCarthy and Arkenhead (1946), Ritter and Nussbaum (1944), and Ritter (1945). Ritter and Nussbaum (1944) reported on cotton fever and stated that "by far the most common occupational disease in Mississippi is one that has received almost no notice from our physicians . . . . It is an acute illness that is referred to by a number of different names—cotton fever, cardroom fever, dust chills or cotton colds being most common."

A Microbiologic Basis for the Etiology of Byssinosis

Prior to 1940 much of the research seeking to identify the etiology of byssinosis focused on either toxic constituents of the plant (MacDonald and Maitland 1934) or on release of endogenous mediators such as histamine (Haworth and MacDonald 1937). In most studies the disease mechanism was postulated to have an immunologic basis (Prausnitz 1936). However, Neal et al. (1942) described an acute illness among workers making mattresses with low-grade cotton and concluded that the illness was caused by inhalation of gram-negative bacteria (GNB) or an associated product contaminating the cotton. This report had a major impact for two reasons. First, it was published in one of the more prestigious scientific journals, *The Journal of the American Medical Association*, and therefore gained wide recognition. Second, rather than associating the respiratory response to "cotton dust," the authors identified a specific bacterial contaminant within the dust as the potential cause of the reaction. Another notable aspect of this report was the association of symptoms with a specific cotton classed

as graded or tinged. This association corroborated with reports linking an outbreak of respiratory disease in 1936 in a textile mill in North Carolina to a "tawny" colored cotton containing much foreign material (Trice 1940).

The association of bacteria with the acute response to cotton dust and with specific fiber properties led to studies investigating the microflora of cotton. For example, Clark et al. (1947) reported that contamination of cotton in the field with GNB is dependent on environmental factors that influence the stage of boll maturity at the time of frost. During the 1940's several other organic dusts were evaluated and an association was found between GNB in these dusts and acute respiratory illness, including grain fever, hemp fever, and illness of workers handling flax or exposed to bagasse (Purdon 1903, Light 1918, Koelsch and Lederer 1933). Schneiter et al. (1948) were the first to describe *Aerobacter cloacae* endotoxin as a possible factor in the etiology of bagassosis.

Research in the 1950's in the United States and the United Kingdom focused more on the definition and incidence of byssinosis rather than on defining the etiology. In 1950 Schilling et al. (published in 1963) developed a subjective basis for classifying the response to cotton dust. They defined the following four stages of response to cotton dust:

Stage	Clinical Response
1/2	Symptoms on some Mondays
1	Symptoms every Monday but not on other days
2	Symptoms every Monday and on other days
3	Symptoms of stage 2 with evidence of permanent respiratory disability

Later, the Organizing Committee (1971) proposed an objective grading scheme for byssinosis based on measurements of pulmonary function. Workers are now routinely tested for their response to cotton dust by pulmonary function testing and a questionnaire that is based on the subjective evaluation of Schilling.

In 1960 endotoxin and GNB were again suggested for being responsible for the response to cotton dust (Pernis et al. 1961). However, other investigators were not convinced and claimed that endotoxins were not involved in the chronic response or at best caused mill fever, a response considered by many not to be related to byssinosis (Antweiler 1961). Toward the latter part of the 1960's and throughout the 1970's and 1980's, the list of potential candidates as causative agents of byssinosis grew to include plant components such as laciniline C–7 methyl ether, tannin, and terpenoid aldehydes; applied toxic materials such as pesticides and desiccants or defoliants; and microbiological contaminants including viable organisms such as gram-positive and GNB and fungi and associated metabolites (including proteases, peptides, mycotoxins, and endotoxins).

While it is unlikely that a single compound is responsible for all of the respiratory effects caused by cotton dust, the most convincing evidence of a role for a single compound comes from several studies that demonstrated a relationship between the acute respiratory symptoms and exposure to aerosols of GNB or endotoxins (table 1). Using either volunteers or cotton mill workers, these studies have demonstrated a dose-response relationship between endotoxin or GNB and chest tightness or changes in forced expiratory volume in 1 second (FEV_1). The most convincing study on the effects of endotoxin was done by Castellan et al. (1987). They demonstrated that airborne endotoxin was a more reliable predictor of the acute response to cotton dust than airborne dust.

Clearly FEV_1, bacteria, and bacterial products in cotton dust cause an acute respiratory response. However, many questions concerning the role of bacteria or bacterial products need to be addressed. What is the mechanism by which endotoxin causes acute pulmonary toxicity? What is the relationship between the bacteria or bacterial products associated with the acute response and the development of a chronic impairment caused by cotton dust? Is there more than one type of microbial product involved in the acute or chronic response to cotton dust? What synergistic actions are involved in the

Table 1. Studies demonstrating a relationship between airborne endotoxin or GNB and symptoms of byssinosis

Subject	Environment	Symptom	Reference
Workers	Mattress manufacturing	Respiratory	Neal et al. (1942)
Volunteers	Experimental	FEV_1	Pernis et al. (1961)
Volunteers	Experimental	FEV_1	Cavagna et al. (1969)
Workers	Cotton mill	Chest tightness	Cinkotai et al. (1977)
Workers	Cotton mill	Chest tightness	Cinkotai and Whitaker (1978)
Workers	Cotton mill	FEV_1	Rylander et al. (1979)
Workers	Cotton mill	FEV_1	Rylander et al. (1983)
Volunteers	Experimental	FEV_1/Chest tightness	Hagland and Rylander (1984)
Volunteers	Experimental	FEV_1	Castellan et al. (1984)
Workers	Experimental	FEV_1	Rylander et al. (1985)
Volunteers	Experimental	FEV_1	Rylander et al. (1986)
Volunteers	Experimental	FEV_1	Petsonk et al. (1986)
Volunteers	Experimental	FEV_1	Castellan et al. (1987)
Workers	Cotton mill	FEV_1	Kennedy et al. (1987)

response to cotton dust? Assuming that microorganisms or their metabolites are involved in the response to cotton dust, questions arise as to strategies for intervention. Can methods be developed to allow the decontamination or inactivation of the microbial products on cotton without damaging the fiber? Can methods be developed to suppress or inhibit the growth of toxigenic microorganisms in the field? To answer these questions, we will need a better understanding of the microbiology of cotton.

Economic Basis for Studying the Microbiology of Cotton

Fiber Quality

The economic basis for understanding the microbiology of cotton relates to the effect of microorganisms on fiber quality and fiber production. Fiber production and quality can be affected by both the natural flora of cotton and microorganisms that are specific pathogens for cotton. The term *fiber quality* refers to properties of the fiber that can affect the quality of the end product or determine the end use of the fiber. These characteristics include fiber length, fiber strength, color, and a measure of fiber fineness (mass per unit length) and maturity (degree of fiber wall development) called micronaire. Changes in fiber color have been associated with field weathering and microbiologic activities of natural flora. Morey et al. (1982) reported that higher levels of GNB were associated with greater amounts of yellow color. The early reports of Marsh and Bollenbacher (1949) suggested that grayness of fiber was associated with higher levels of fungi.

Microbes have also been associated with the degradation of fiber strength. For example, fungi are capable of causing damage to the cellulose (Marsh et al. 1949). Such contamination may affect the micronaire of the fiber since fiber maturity, a component of micronaire, is a function of cellulose degradation. Such damage will affect not only the processability of the fiber but will result in a lower quality end product. Clearly more research is

needed to understand the influence of normal microbial flora of cotton on fiber quality.

Fiber Processing

The ability to process cotton into yarn and fabric is influenced by a variety of variables, including fiber quality, cultivation and harvest conditions, and moisture content. The potential for microorganisms to influence each of these variables and thereby influence fiber processing has not been adequately evaluated. One problem related to fiber processing that is of current interest to the textile industry and that may be affected by microbes is sticky cotton. Sticky cotton results from the accumulation of a sugar-containing sap on the fiber. This sap is called honeydew and is thought to originate either from an endogenous source (Bourdy 1980), such as the excretion products of aphids and white flies (Heuer and Plaut 1985), or from microbial sources such as corynebacteria (Wyatt and Heintz 1982). Processing such cotton causes the fiber to stick to the rollers of the equipment, requiring frequent cleaning, slowing production, and degrading yarn quality.

Sticky cotton has become one of the major concerns of the textile processing industry since 1980. A better understanding of the microbiology of cotton may help solve the problem. If microorganisms such as the corynebacteria contribute to the accumulation of sugar on sticky cotton, then a better understanding of the ecology of fiber colonization by corynebacteria or other suspect microbes may provide a basis for control. Alternatively, if microbes are not the source of excess sugars on cotton, they can be used to reduce excess sugars on cotton. Cottons have been sprayed with selected microorganisms or treated with nutrients to increase microbial degradation of sugars (Balasubramonya et al. 1985). While these studies are promising, much work remains to be done to understand the relationship between microbial colonization of cotton and its processability and the development of intervention strategies to improve processing.

Fiber Production

Cotton is susceptible to a variety of diseases caused by both bacterial and fungal pathogens, and the economic impact of these diseases on fiber production and quality is well documented. To better understand the interaction of microbial pathogens with the plant, basic information is needed about the organism and the plant's defense (resistance) mechanism. This information is usually obtained by laboratory studies designed to evaluate the life cycle of the organism and the interaction between the plant and the pathogen, and then by field studies to coordinate laboratory data. By understanding the pathogen-microbe interface, methods may be developed to intervene either by preventing or reducing pathogen contact with the host or by reducing damage on contact by enhancing plant resistance. Better understanding of the pathogen-host ecology may provide guidelines that allow development of predictive models. Such models have been developed for beans, allowing the prediction of disease incidence on the basis of pathogen population on individual leaves (Rouse et al. 1985). Valid models would provide guidelines for the timing of appropriate intervention strategies.

The impact of the natural flora of cotton on fiber production is not as well understood as the impact of cotton pathogens on production. However, an understanding of the basic ecology of the natural flora on cotton may provide new strategies for enhanced production, improved fiber quality, or the reduction of toxic material on the fiber. Such ecologic studies may provide new methods of controlling old problems. For example, on strawberries and potatoes bacteria can be used to prevent early season frost damage. Selected species of GNB (*Pseudomonas fluorescens*, *Pseudomonas syringae,* and *Erwinia herbicola*) are capable of ice nucleation. Ice-nucleating bacteria initiate the formation of ice crystals at a higher temperature than non-ice-nucleating species and increase frost damage at temperatures that otherwise would not cause damage. Non-ice-nucleating strains of bacteria have been used in field studies to determine whether these strains can compete with ice-nucleating strains and reduce damage from frost (Lindow 1983).

Many of the studies discussed in this book have sought to remove endotoxin-producing bacteria from cotton. These and other studies yet to be proposed may provide a means to control the microbes that have a detrimental effect on public health or the economic development of cotton.

Application to Other Environments

Much of the knowledge gained in researching the interaction of bacteria and other microorganisms with cotton can be applied to other environments. For example, it is unlikely that the role of endotoxin in the respiratory response to cotton dust is unique. Cotton is just one of many vegetable fibers that have been documented to cause acute and chronic respiratory responses. Furthermore vegetable fibers are just one category of materials from which organic dusts are generated. As with vegetable fibers, there are a variety of environments in which exposure to organic dusts are associated with acute and chronic respiratory disease. Such environments include animal confinement facilities, indoor facilities (closed environments), and biotechnology research and production facilities. The research done to understand the microflora on cotton is also applicable to problems encountered in these areas.

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Chapter 2. Botanical and Microbiological Contaminants of Cotton Fiber: Early Investigations

Philip R. Morey

Prior to 1973 little quantitative information was available on the botanical composition of raw cotton trash or dust. An experiment by Corley (1966) showed that leaves were an important component of seed cotton trash. Kearney (1929) observed that bracts were present in raw cotton received at the textile mill. Studies on extracts of whole bracts (Nicholls et al. 1966) showed that this plant part contained agents possibly involved in the etiology of acute byssinosis. Because of the work of Bouhuys and coworkers (Nicholls et al. 1966) most attention in the early 1970's focused on bracts as a reservoir for the etiologic agent of byssinosis.

The studies mentioned in this chapter began in 1973 after a suggestion that Shirley analyzer wastes (a Shirley analyzer is an instrument used to determine the nonlint or nonfibrous content of cotton samples) collected during gin lint cleaning would be useful for quantifying the amount of bracts and other plant parts in raw cotton (Wakelyn and Sasser, personal communication 1973). An initial study (Morey et al. 1975) showed that bracts and other plant parts could be identified with certainty among vegetable trash particles larger than about 500 micrometers (μm). Studies were designed and performed to meet the following objectives:

1. To identify plant parts larger than about 500 μm in raw cottons of different geographical origin and in cotton process streams in cottonseed oil mills and garnetting plants
2. To estimate the composition of plant parts larger than about 50 μm in raw cottons and in process streams in cottonseed oil mills and garnetting operations
3. To determine the fragmentation potentials of plant parts in raw cottons and to estimate the botanical components likely to be present in dusts during processing of raw cotton
4. To determine whether field and climatic variables affect the concentration of gram-negative bacteria on various parts of cotton plants
5. To use the classing system of the U.S. Department of Agriculture-Agricultural Marketing Service (USDA–AMS) as a means for estimating the plant part and gram-negative bacterial concentrations in raw cotton of various grades and color groups.

Exact Identification of Plant Parts in Cotton Materials

Plant Parts from Raw Cotton

Visible wastes from the ginned lint of an Acala cultivar that had undergone from zero to three cleanings were separated into size categories on a sieve stack containing mesh openings of 250, 421, 841, 1,410, and 2,000 μm (Morey et al. 1975). Particles in each size fraction were separated by morphology with the aid of a stereomicroscope into 11 botanical types, namely bracts, leaves, vein materials, petioles, exocarps, mesocarps, endocarps, bark, wood, seed coats, and cotyledons (see Morey 1982 for definitions).

Representative kinds of plant parts of each morphological group were embedded in paraffin or plastic, sectioned, stained, and examined anatomically for confirmation of plant part identity. It was determined, for example, that among 24,730 plant parts collected on the 841-μm mesh sieve screen (particles in the size range of 841–1,410 μm) the following rank order of particles from the most to the least abundant occurred: bracts, leaves, petioles, seed coats, capsules (three pericarp components combined), vein materials, wood, bark, and cotyledons.

These initial plant part studies on visible wastes from a Shirley analyzer were continued using raw cottons that had either been derived from machine-stripped or machine-picked cultivars and from cultivars that had been treated with defoliant chemicals. Only those plant parts that did not pass through an 841-μm mesh opening (or larger mesh sieve screens) were identified in these studies.

The percent weight content of bracts, leaves, and weeds (mostly silverleaf nightshade and gramineous weeds) in four machine-picked and two machine-stripped raw cottons is presented in table 2. Bracts were the predominant trash component in these raw cottons. Weed trash was significant (up to 34 per-

Table 2. Visible plant wastes in Shirley analyzer wastes from raw cottons

Plant part	Plant waste (percent)* From machine-stripped cultivars	From machine-picked cultivars
Bracts	33–42	38–52
Leaves	5–6	14–26
Vein material†	4–5	6–12
Weeds‡	17–34	2–21

* Range of plant part composition. Plant part sizes ranged from 841 to 2,000 μm.
† Cotton bracts and leaf veins.
‡ Plant parts from monocotyledonous and dicotyledonous weeds initially entrained in cotton during machine havest.
Source: Morey et al. (1976b).

Table 3. Plant waste from linters and willowed picker

Plant part	Plant waste (percent)* From linters	From willowed picker
Bracts	<0.1	8.8
Leaves	0.7	2.6
Vein material	ND†	2.2
Seed coats	80.6	37.1
Funiculi	1.3	6.0

*Particle size greater than about 500 μm.
†*ND*, None detected.
Source: Morey and Wakelyn (1976).

cent of visible wastes) in cotton that had been machine-stripped. Cotton leaf blades were a major component (minimum content of about 15 percent) of plant trash derived from machine-picked cultivars.

An additional study on machine-picked cottons showed that leaf blades can be as abundant as bracts in Shirley wastes derived from nondefoliated cultivars (Morey et al. 1977). Leaf particles were only about half as abundant as bracts in cottons that had been treated with chemical defoliants prior to machine picking.

The plant trash materials present in Shirley wastes from lint before and after one stage of gin lint cleaning were quantified (Morey et al. 1976a). The content of bracts and leaves in visible Shirley wastes increased from 45 to 55 percent following lint cleaning. These results showed that bracts and leaves are more difficult to remove than other plant parts during mechanical cleaning of lint.

Plant Parts in Cotton Materials From Oil Mills and Garnetting Plants

The type of plant trash (particles larger than 500 μm) in cotton materials from oil mills and garnetting plants was studied and compared to that in raw cottons (Morey and Wakelyn 1976). Botanical trash composition among identified plant parts in nontextile cotton materials differed considerably from that in raw cotton. In one linter sample (from an oil mill), less than 0.1 percent of the plant parts examined were bracts (table 3). As might be expected from a product from a cottonseed oil mill, most of the plant parts in linters were seed coat fragments from the cottonseed itself. On the other hand, the bract content in one garnetting raw material (willowed picker) was approximately 8.8 percent; seed coat fragments accounted for 37.1 percent of the plant parts (table 3). These early studies suggested that the vegetable composition of dusts in different cotton industries should differ significantly.

Weeds and Grasses

The presence of weeds in raw cotton (Morey et al. 1976b) led to the development of a taxonomic procedure to determine weed particle origin (Jaco et al. 1978). The epidermal surface characteristics of weed particles as seen in a stereomicroscope or by reflected light microscopy are sufficiently unique so as to allow for generic and sometimes species identification of some of the particles. This procedure has been used on a Coker raw cotton from South Carolina to show that morning glory (*Ipomea* sp.) particles accounted for about 25 percent of the

weed particles in the harvested cotton (Jaco et al. 1978). Stems and leaves from silverleaf nightshade (*Solanum elaeagnifolium*) comprised about one-sixth of all trash particles in a Paymaster 111 raw cotton from Texas.

Classers at the USDA–AMS may lower the grade of a sample by one or more divisions because of the presence of "grass" particles. Examination of 198 grass particles from three classing offices showed that most were gramineous weeds (Jaco et al. 1978). For example, crabgrass and crowfoot grass plant parts (*Digitaria* and *Dactyloctenium* sp., respectively) dominated the particles identified as grass in the Raleigh, NC, USDA–AMS office.

Classers at the USDA–AMS may also lower the grade of raw cotton because of the presence of "bark" particles. Most bark particles in raw cotton were identified anatomically as fiber groups from the primary phloem of cotton plant parts (Morey et al. 1978). These plant parts in raw cottons are thus anatomically analogous to the "fiber" of the flax (*Linum usitatissimum*) plant (flax fibers are derived from the pericyclic region of the stem).

Limitations in Identifying Plant Parts

The exact identification of plant parts by anatomical study is confounded by the minimal diagnostic size of tissue systems needed for ascribing a particle to one or another plant part category. For example, the thickness of the bract lamina is about 100 to 500 μm (Morey and Raymer 1978). The entire cross section of the lamina (adaxial to abaxial epidermis) must be examined to conclusively identify a plant part as a bract. In practical terms this means that for most plant parts it is not possible to use anatomical methods alone to identify particles of a size less than about 500 μm.

One exception to the diagnostic size limitation needed for identification is the capitate hair on the epidermal surfaces of bracts and leaves. These hairs are present in varying density on the epidermal surfaces of bracts and leaves and are thought to function as reservoirs for chemicals such as flavonoids and tannins. Examination by scanning electron microscopy of bract particles from a bale of strict low middling cotton showed that about 3 to 7 capitate hairs (size of each hair was about 20 to 35 μm x 45 to 50 μm) were present per square millimeter (mm^2) of bract surface (Kosmidou-Dimitropoulou et al. 1980). A bale of strict low middling cotton with the equivalent amount of about 21,000 entire bracts (surface area of approximately 1,600 mm^2 per bract) thus contains approximately 1.23 to 2.54 x 10^8 capitate hairs from bracts alone. These capitate hairs may be a source of chemical agents potentially important in the etiology of byssinosis.

Estimation of Plant Part Composition in Cotton Materials Based on Particle Morphology

Studies in the late 1970's (Morey 1979 a and b) estimated the plant part composition for particles as small as 50 μm in raw cottons and in nontextile cotton materials. A methodological difference in this study compared to previous studies was that plant parts in the size range of 50 to 500 μm were assigned to one or another botanical category based on morphology and not on anatomical characteristics. Since it is impossible at this small particle size to anatomically differentiate between cotton leaf, cotton bracts, and weed leaves, all kinds of laminar (flat) particles were combined into one morphological category, namely "leaflike" trash. Morphological characteristics were also used to categorize other 50- to 500-μm size particles into botanical fractions such as seed, endocarp, exocarp-mesocarp, bark, and stem trash.

A study of the plant part composition of 71 raw cottons from the grade division good middling through good ordinary showed that significant differences in the content of leaflike trash occur between USDA–AMS grade divisions (Morey 1979b; see also table 4). The low middling grade division contains about five times more leaflike trash than the higher quality cottons such as middling. Seed, bark, and stem trash content, however, did not differ significantly (Morey 1979b).

Morphological techniques were used to estimate the plant part composition of nontextile cotton materials, and some of these data are presented in table 4. Cottonseed at the oil mill after cleaning contained only 0.03 percent leaflike trash, or an amount one to two orders of magnitude less than that in the common grade divisions of raw cotton. A sample of

Table 4. Plant waste in raw cottons, cleaned cottonseed, and second-cut linters

Cotton material	Plant waste (pecent)*			
	Leaf bracts and weeds	Seed fragments	Stem	Bark
Middling raw cottons	0.40	0.92	0.07	0.12
Strict low middling raw cottons	0.85	1.01	0.18	0.22
Low middling raw cottons	2.02	1.39	0.47	0.23
Cottonseed after cleaning	0.03	0.27	0.38	NI†
Second-cut linters	0.2	46.47‡	ND†	NI

*Identity of plant parts larger than about 50 μm estimated by morphology in stereomicroscope or in reflected light microscopy.
†*ND*, None detected. *NI*, No information.
‡Considerable amounts of linters were attached to seed coat fragments.

Sources: Morey (1979b, 1981).

second-cut linters contained 0.2 percent leaflike trash and 46.5 percent seed coat fragments. Morey (1979a) also found similar results to those shown in table 4, concluding that major differences in terms of plant part composition occur in cotton materials used in the textile and nontextile industries.

Fragmentation Potentials of Cotton Plant Parts

Whole Bracts

Bracts were collected from greenhouse-grown Tamcot SP–21 at postanthesis intervals of 0 to 120 days. Anatomical studies showed that lignification of bract tissues was complete at 20 to 30 days after anthesis, and mesophyll tissue collapsed (cells lyse and bracts dry out) at about 80 days after anthesis (Morey and Raymer 1978). Fragmentation studies (in which whole bracts were abrasively ground in a roller mill and then arbitrarily separated into size classes greater than and less than 250 μm) showed that the friability of whole greenhouse-grown bracts increased to a maximum about 80 days after anthesis.

Fragmentation studies were later carried out on whole bracts collected in the field at intervals before and after the first killing freeze (Fischer et al. 1982). Bracts were abrasively milled and subsequently sieved on a series of screens with mesh openings from 10 to 106 μm. Analytical results showed that the mass median diameter of bract particles derived from whole bracts of a Paymaster 909 cultivar was 19 μm just before the first freeze and 13 μm after the freeze (Fischer et al. 1982). Freezing and drying, thus, have a strong effect on the friability of this plant part.

Bract Plant Parts

Some anatomical differences have been found between bract particles of the same size collected from raw cottons and from garnetting raw materials (Morey and Wakelyn 1976). Bract particles from raw cottons contained more lamina tissue and fewer lignified veins (lignified xylem tissue) per plant part surface area than bract particles found in willowed picker used in garnetting. In other words, bracts from willowed picker were more woody or stemlike than similar plant parts from raw cotton.

A fragmentation test was subsequently carried out on similar-size (500 to 999 μm) 15-mg lots of leaflike trash from garnetting and raw cotton wastes (Morey 1979a). Results showed that leaflike plant parts (bracts, leaves, veins, and weed fragments) from garnetting materials such as picker, mill motes, and gin motes were more resistant to abrasion and fragmentation than similar-size plant parts from strict low middling and low middling raw cottons.

These results suggested that the leaflike plant parts from garnetting raw materials can be expected to generate less-fine particulate matter during mechanical processing than leaflike plant parts from raw cottons.

Botanical Composition of Raw Cotton Dust

The botanical components in raw cotton dusts of less than 10 μm were estimated from the type of data presented in tables 4 and 5 (Morey 1979b). In fragmentation experiments, 1-g lots of each plant part (size 500 to 999 μm) were abrasively milled, and plant part debris smaller than 10 μm was recovered and quantified. It is important to note that the 1-g lots were not derived from raw cottons but rather from whole plant parts collected in the field, from seed cotton, or from other sources. Therefore, bract plant parts used in fragmentation experiments were derived from whole bracts that had been hand picked and ground up to yield a large fraction of particles 500 to 999 μm in size. Bark, stem, and capsule materials used to make plant parts 500 to 999 μm in size originated from larger pieces of stems and capsules from seed cotton trash of an Acala 1517 cultivar from New Mexico.

Fragmentation tests were carried out on 1-g lots of the following plant parts: bracts, leaves, leaf petioles, silverleaf nightshade leaves, bark and wood from cotton stems, endocarps, exocarps-mesocarps, and seed coats. The amount of each plant part type that was ground up into less than 10-μm particles by an equivalent amount of abrasion is presented in table 5 (Morey 1979b). Bracts were the most friable plant part. Stem materials, including the petioles and wood, were nearly as friable as the bracts.

An estimate was then prepared of the botanical components present in raw cotton dust of less than 10 μm. This estimate was based on the fragmentation potentials in table 5 and the amount of each plant part (size greater than about 50 μm) estimated to be present in gross botanical trash in raw cottons. Uncertainties in this estimation process include the following: (1) the leaflike trash in raw cottons was partitioned 58%:22%:20% among bracts, leaves, and weeds, respectively, based on earlier work on visible Shirley analyzer wastes (see table 2 in Morey et al. 1976b); (2) the percent weight composition of plant parts in the size range of 50 to 500 μm is based on surface morphology characteristics and not on anatomical certainty; (3) plant parts used in fractionization tests were not obtained from raw cottons.

The estimated percentage by weight of botanical components present in raw cotton dusts of less than 10 μm from middling through low middling grade divisions is presented in table 6. Although many technical uncertainties are involved in these estimates, they nevertheless are the only data available on the subject. Leaflike and stem components collectively account for more than 80 percent of the likely botanical components in raw cotton dusts. This is due primarily to the finding that bracts, wood, and petioles are readily broken apart to a size less than 10 μm during fragmentation tests (table 5).

Table 5. Percent of plant parts that were ground up into particles 10 μm or less in size after an equivalent amount of abrasion

Plant part	Percent of plant waste ground to 10 μm or less
Bracts	33
Wood	29
Petioles	18
Exocarps-mesocarps	15
Bark	7
Endocarps	4
Leaves	4
Silverleaf nightshade	2
Seed coats	0.2

Source: Morey (1979b).

Table 6. Estimate of botanical ingredients in raw cotton dusts from various common grades

Plant part	Raw cotton dust* (percent)
Leaves, bracts, and weeds	70–71
Stems	14–18
Capsules	4–8
Bark	3–7
Seed	0.5–2

*Dusts 10 μm or less in size from middling, strict low middling, and low middling cottons.

Source: Morey (1979b).

Tracer Studies

It was anticipated in early botanical studies that the origin of vegetable components of raw cotton dust could not be determined solely through use of anatomical and morphological techniques. This is because cell wall fragments, whole cells, and groups of cells from most plant parts are remarkably indistinguishable by light microscopy. A fluorescent tracer technique was suggested as an approach to determine the origin of raw cotton dusts (Morey et al. 1975). The technique uses a dye, color index basic yellow 37 (BY37), to label plant parts in the dust. Studies showed that whole leaves from a greenhouse cultivar could be labeled with aqueous solution of this dye, and subsequently leaf fragments as small as 2 to 10 μm could be recognized due to their characteristic yellow fluorescence in reflected light microscopy (Morey and Raymer 1978).

A group of leaf particles totaling 270 g and having a median particle diameter of 64 μm was prepared from leaf blades of a Coker cultivar that had been dyed with BY37 (Morey et al. 1981). All the leaf tracer was manually added into one fourth of a bale of middling raw cotton which was then carded in the model cardroom at North Carolina State University, Raleigh, NC. Pneumafil filter wastes collected during the carding of this cotton contained BY37-dyed leaf particles with a median diameter of 54 μm. BY37-dyed leaf particles were also clearly recognizable in elutriated dusts collected in the cardroom. Unfortunately, experiments to compare the relative fractionization potential of dyed bracts, leaves, and other plant parts were not performed.

Gram-Negative Bacteria on Cotton Plant Parts

Whole Plant Parts

Early studies showed that an amplification of gram-negative bacteria (GNB) of about one or two orders of magnitude occurred on bracts when this plant part senesced or when green bracts were killed following a freeze (Morey et al. 1983). GNB per gram of field bract increased from just under 1×10^6 to over 1×10^8 within 1 wk after the first freeze (Fischer et al. 1982). This amplification was likely associated with the sudden availability of nutrients from lysed plant cells. The increase in friability of postfreeze bracts (Fischer et al. 1982) may have been due to microbiologically induced deterioration of bract cells and tissues.

Levels of GNB, however, do not increase on all cotton plant parts following senescence from freezing (Morey et al. 1983). The number of GNB per gram of leaf blades remained at about 1×10^5 from a period of about 6 wk before to 6 wk after the first freeze (Fischer et al. 1982).

Plant Parts in Classers Samples

Experiments in which all plant trash particles were removed from a light spotted color grade of raw cotton showed that botanical trash collectively contained about one order of magnitude more GNB per gram than lint that was devoid of botanical trash particles of a size larger than about 50 μm (Morey and Rylander 1982). The concentration of GNB and endotoxin were determined in 296 USDA–AMS classer samples that included all common grade divisions and color groups from the 1980 crop (Morey et al. 1984). Samples from poorer grade divisions and from spotted and tinged color groups consistently had higher contents of GNB and endotoxin than better grades.

The plant parts (size greater than 50 μm) present in 70 of the 296 classer samples were separated from the lint. Plant parts and lint devoid of trash particles were then examined for GNB and endotoxin con-

tents. Analyses showed that leaflike trash (bracts, leaves, and weeds) contained about one order of magnitude more GNB per gram than found on unstained (white) lint devoid of trash particles larger than 50 μm (Fischer et al. 1986). Stained lint that could be separated from white lint contained a concentration of GNB similar to the concentration found on leaflike trash.

Fischer et al. (1986) showed that 67 percent of the GNB and 89 percent of the endotoxin on this group of 70 raw cottons were present on the white lint (cleaned lint represented more than 97 percent of the sample weight). It is possible, however, that plant particles less than 50 μm contaminated the fiber and thus accounted for some of the GNB and endotoxin found in the cleaned lint. The results from these botanical and microbiological studies with classers' cottons nevertheless suggest that even if all large botanical trash could be removed from raw cotton by mechanical cleaning the content of GNB and endotoxin in or on the fiber would not be greatly reduced.

Research Recommendations

Fractionation of Plant Parts

1. Repeat experiments (Morey 1979b) in which plant parts are milled to generate particles of a size less than 10 μm.
2. Use plant parts derived solely from raw cottons.
3. Conduct milling tests using small amounts (10 to 15 mg samples) of plant parts—perhaps using the method in Morey (1979a).

Tracer Studies

Consider using radioactive label (carbon 14) as a tracer to compare each plant part type with regard to ability to generate respirable dust. Small amounts of each type of labeled plant part could be added to the cotton process stream such as in a miniature gin or card. Dusts could be collected with filter samplers. This approach should be less labor intensive than that used previously (Morey et al. 1981) with BY37 dye on leaves. Additionally, the use of radioactive label overcomes the objection that dyeing of plant parts in an aqueous solution changes the friability of the plant part being studied.

Cotton Leaf and Bract

Explore differences between leaves and bracts with regard to gram-negative bacterial colonization (bacteria do not amplify greatly on leaves) noted in previous studies (Fischer et al. 1982, Morey et al. 1983). Such studies might suggest genetic approaches for making the cotton plant less suitable for gram-negative bacterial colonization.

Flax and Cotton

Explore similarities and differences of cotton and flax plants with regard to the structure of the phloem fibers and the capacity of phloem fibers to support the amplification of GNB. Stem and bark are estimated to contribute approximately 20 percent of the weight of raw cotton dust (Morey 1979b). The phloem fibers in flax and the bacteria growing on these fibers are the likely sources of byssinotic agents in flax dusts. It is unknown at present whether the phloem fibers and perhaps also the wood fibers of cotton make a flax-like contribution to cotton dust components. The possibility that there may be structural as well as microbiological similarities in the dusts from these two different commercial fibers should not be overlooked.

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Chapter 3. Bacteria and Fiber Quality

Preston E. Sasser

The relationship between the presence of microorganisms on raw cotton fibers (ginned lint) and the quality of the fiber has been studied for many years. Marsh et al. (1951, 1958) worked to determine the influence of weathering on the properties of cotton fibers. These studies focused attention on the fungi found on the fibers. Weathering was found to cause a significant increase in the pH of aqueous extracts of the fiber, and pH levels were generally higher for the lower grade (darker color) cottons. Lower grades, lower fiber length and strength, and lower yarn strength were reported for the field-weathered cottons. No quantitative measurements were made on the fungi; therefore, no correlations were given. Marsh (1955) reported elevated pH levels in lower grade (grayer) cottons from the 1954 U.S. crop.

Hall and Elting (1951) reported on a condition they called cavitoma, whereby the cotton fibers were damaged by the action of microorganisms and yet the color of the cotton had not changed. They demonstrated fiber damage when cotton samples were conditioned for up to 2 mo at 75 percent relative humidity. They reported the presence of both bacteria and fungus in their samples of "cavitomic" cotton. They found pH values in the range of 8 to 10 in samples with "advanced cavitoma."

During the 1970's, interest was focused on the bacteria found on raw cotton fibers because of the problems that occurred with cotton dust and byssinosis in textile mills that processed cotton. A few researchers reported fiber quality data. However, no relationships between quality and the presence of bacteria were strong enough to use any of the quality factors as a predictor of the presence of bacteria on the fibers. Higher levels of bacteria were generally found on finer fibers and lower grade and lower maturity cottons. Most of the studies reported in the 1970's and 1980's concentrated on gram-negative bacteria (GNB) because of the suspected role of endotoxins (toxins associated with the bacteria) in the acute response of humans to inhaled cotton dust.

Fischer and Morey (1979) used cottons from the 1977 U.S. crop to investigate the use of classer's grade as a predictor of the presence of gram-negative rods in raw cotton. They reported higher levels of GNB in the lower grades of cotton that had more yellow color in the fiber. They also found higher levels of GNB and endotoxins in the trash of the raw cotton and higher levels of GNB in grades having a higher trash content. No regression or correlation analyses were given.

Simpson and Marsh (1982) studied 143 samples of commercial cotton samples from the 1980 U.S. crop. They reported wide sample-to-sample variations in the counts for total bacteria and GNB. Their counts of total bacteria ranged from 10×10^4 to 290×10^6 per gram of fiber. They reported especially high counts in some cotton samples of spotted and tinged grades. No correlations were made between the grade codes and the bacterial counts. The authors felt that no interregional comparisons for bacterial count could be made from their data.

Morey et al. (1980) reported the gram-negative bacterial content and micronaire on 12 samples of raw cotton that represented the combinations of

Table 7. Gram-negative bacterial content and micronaire of different color groups and grades of raw cotton

	Bacterial content (millions of colony forming units/g) and micronaire (in parentheses)			
Grade	White	Light spotted	Spotted	Tinged
Middling	0.4 (4.0)	34.0 (3.0)	79.0 (3.2)	160 (2.2)
Strict low middling	7.8 (4.5)	4.7 (4.0)	3.6 (3.8)	150 (2.3)
Low middling	10.0 (3.8)	8.2 (3.7)	44.0 (3.7)	86 (2.6)

Source: Morey et al. (1980).

grades within the fiber color groups (table 7). They concluded that GNB appear to be negatively related to micronaire in these samples. No statistical analyses were reported, but it also appeared that the more yellow cottons (spotted and tinged color groups) contained higher levels of GNB.

Gram-Negative Bacterial Content and Fiber Properties

Fischer et al. (1980) and Morey et al. (1980, 1982) were among the first researchers to include a broad range of fiber properties in their studies relating to GNB. The gram-negative bacterial content was studied by Fischer on 109 samples from the 1978 U.S. upland crop and by Morey on 255 samples from the 1979 crop. Both studies included data on fineness (fineness and denier), maturity (maturity ratio, percent of mature fibers, and causticaire maturity), and classers' grade and also included data determined by the High Volume Instrument (HVI) System (micronaire, upper-half-mean length, length uniformity index, strength, trash grade, grayness, and yellowness).

Table 8 gives a summary of the correlation coefficients found in these two studies between the fiber properties and the bacterial content. All of the correlations in table 8 are statistically significant at the 0.1 percent level except for those for grayness and trash grade.

These studies of samples from the crops of 1978 and 1979 are remarkable in their agreement. In general, the data agree in that higher quality cottons have lower levels of GNB. The grayness of the fiber does not seem to be related to the gram-negative bacterial content, but the levels of GNB are higher in the more yellow samples. Fiber samples with longer length, higher length uniformity, higher strength, and higher micronaire have lower levels of GNB. Similarly, more mature and more coarse fibered cottons have lower levels of these bacteria. This could be the result of lower surface area per gram of fiber for these cottons.

Endotoxin Content and Fiber Properties

Morey et al. (1982, 1984) also reported correlations between the endotoxin from GNB and fiber properties. These studies used samples from the 1979 and 1980 U.S. upland crops. Table 9 summarizes the correlation coefficients between endotoxin levels

Table 8. Correlation coefficients between gram-negative bacterial content and fiber properties

	Correlation coefficients	
Fiber properties	1978 Crop*	1979 Crop†
Grayness	0.06	0.08
Yellowness	0.61	0.56
Trash grade	0.01	-0.05
UHM length	-0.46	-0.55
Length uniformity	-0.53	-0.45
Strength	-0.36	-0.42
Micronaire	-0.71	-0.50
Maturity ratio	-0.64	-0.55
Percent mature fibers	-0.66	-0.56
Causticaire maturity	-0.66	-0.56
Fineness	-0.55	-0.28
Denier	-0.55	-0.28

*From Fischer et al. (1980).
†From Morey et al. (1981).

Table 9. Correlation coefficients between endotoxin content and fiber properties

	Correlation coefficients	
Fiber properties	1979 Crop*	1980 Crop†
Grayness	-0.02	0.59
Yellowness	0.51	0.23
Trash grade	-0.07	0.37
UHM length	-0.42	-0.18
Length uniformity	-0.38	-0.14
Strength	-0.32	0.01
Micronaire	-0.37	-0.23
Maturity ratio	-0.43	-0.30
Percent mature fibers	-0.44	-0.30
Causticaire maturity	-0.44	-0.30
Fineness	-0.21	-0.14
Denier	-0.21	-0.14

*From Morey et al. (1982).
†From Morey et al. (1984).

and the various quality factors. The correlation coefficients from the studies of Morey et al. (1982) are statistically significant at the 0.1 percent level except for those for color grayness and trash grade. The coefficients from Morey et al. (1984) are significant at the 0.1 percent level except for those for UHM length (1 percent level), length uniformity (5 percent level), fineness (1 percent level), denier (1 percent level), and strength (not significant). The grayness and trash grade factors explain more of the variability in the endotoxin levels in the 1980 crop than in the 1979 crop. All of the other fiber properties are less important.

Grade data were given in a series of studies by Simpson et al. (1984) and Simpson and Marsh (1986, 1988) on the bacterial content of samples from the commercial crops of 1982, 1984, and 1986. No correlation or regression statistics were given for these studies. The data in the studies suggest that the levels of total bacteria and GNB found in a given grade for one crop year do not correspond to the level of bacteria found in that grade for other crop years.

Summary

In general the lower grades of cotton have higher levels of bacteria. However, even a good grade of cotton such as middling can contain more than a million bacteria per gram of fiber. None of the studies showed any correlation between grayness and the total or gram-negative bacterial contents. However, the number of bacteria in a sample was positively related to the yellowness of the sample. The bacterial counts were also related to other quality factors. Longer, stronger, more coarse, and more mature cottons have lower bacteria counts. The fact that fine or immature cottons have higher levels of bacteria may be related to a higher surface area per gram of fiber.

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Chapter 4. The Gram-Positive Bacterial Flora of Cotton

C.E. Heintz and 0.0. Akinwunmi

Considerable information is available about the gram-negative bacteria (GNB) found on lint because of their association with the symptoms of byssinosis in textile workers [as reviewed by Jacobs et al. (1991) and discussed elsewhere in this book]. Less is known about the gram-positive bacterial flora present on cotton, although identification protocols for these organisms have been presented by Tuxford and Hoult in Fischer et al. (1992) and by Akinwunmi (1989).

Information about the gram-positive bacteria is important for several reasons. First, since they constitute part of the normal flora of cotton, being able to recognize and identify them is necessary in order to examine their role in the formation of the bacterial assemblages on cotton. In addition, although it is well known that many gram-negative bacteria (GNB) form yellow or brown colonies (such as *Enterobacter agglomerans*, *Flavimonas oryzihabitans*, and *Pseudomonas syringae*), some of the gram-positive bacteria associated with cotton are similarly pigmented. Being able to differentiate gram-negative from gram-positive organisms may be of use in designing studies to determine the contribution of various types of bacteria to, for example, problems of yellowing and spotting of lint.

Second, alleviating problems associated with airborne endotoxin from GNB may be possible by intentionally introducing gram-positive organisms at boll opening. This introduction may prevent or reduce the colonization of lint by GNB. Field experiments performed by Fischer and Foarde (1991) demonstrated that gram-negative organisms could be displaced by spraying the cotton with gram-positive organisms. In addition, laboratory data suggest that gram-positive bacteria may be more successful competitors than GNB on lint (Wyatt and Heintz, unpublished data). These investigators found that viable counts of GNB were one to several logarithmic units lower if the samples contained substantial numbers of gram-positive bacteria.

Third, information on the effect of temporal or environmental factors may be useful for estimating the population of gram-positive bacteria. Factors such as moisture have been shown to have a large effect on the numbers of GNB on lint (DeLucca et al. 1990, Heintz et al. 1990, Batson et al. 1991, Zuberer and Kenerley 1991, and Fischer and Foarde 1992), and cotton exposed to a lot of rain or high humidity is likely to harbor large numbers of GNB. Knowing which bales came from regions with wet weather at harvest can alert the mills to take measures to protect workers from aerosolized GNB or endotoxin that is likely to form during the processing of such bales. Similar information on the effects of environmental factors on gram-positive bacteria is lacking.

Study Procedures Used To Isolate and Identify Gram-Positive Bacteria

In 1986 and 1987, we designed a study to isolate and identify the gram-positive bacteria found on different parts of Paymaster 404 cotton plants grown at the Texas Agricultural Experiment Station in Lubbock, TX. Samples were collected at weekly intervals between seedling emergence and harvesting of the lint. Ten plants were pulled from the soil and immediately divided aseptically into the following parts (if present): roots, stems, branches, leaves, flowers, unopened bolls, and open bolls (lint, bracts, and seeds were separated later and also examined). Similar parts were pooled into a single sample and transported to the laboratory in an ice chest.

Subsamples were collected from the samples and extracted in fluorescence treponemal antibody (FTA) buffer, from which serial dilutions were prepared for standard plate counts. Only those organisms that could be isolated and cultured aerobically under mesophilic incubation conditions on full-strength tryptic soy agar (TSA) containing cycloheximide (to inhibit the fungal flora) were included in the study. Examples of each morphologically distinct colony that developed on the agar were identified. The plant part(s) on which each colonial type occurred is documented in table 10, although the subculture used for identification came only from the first colony isolated.

The identification of each organism was based on its cellular and colonial morphologies, staining reactions, results of biochemical tests, and fatty acid

Table 10. Gram-positive bacteria and the plant parts on which they were found

Strain numbers*	Species	Plant parts
Sporogenous Rods		
5	*Bacillus brevis*	Roots, stems, leaves, lint
18	*B. cereus*	Roots
10	*B. circulans*	Leaves, lint
47	*B. laterosporus*	Roots
4	*B. licheniformis*	Roots, lint
11	*B. pumilus*	Roots, lint
6	*B. sphaericus*	Stems
13	*B. sphaericus*	Lint
1, 7, 22	*B. subtilis*	Roots, stems, leaves, lint
Asporogenous Rods		
3	*Clavibacter michiganense*	Roots
8	*C. michiganense*	Stems, leaves, lint
15	*C. michiganense*	Leaves
21	*C. michiganense*	Lint
62	*C. michiganense*	Stems
19	*Oerskovia* sp.	Lint
39	*Oerskovia* sp.	Lint, bract
61	*Rhodococcus rhodochrous*	Roots
9	*Propionibacterium* sp.	Lint
12	*Streptomyces* sp.	Roots, lint
Cocci		
29	*Micrococcus agilis*	Branches, leaves, bract
25	*M. luteus*	Leaves, lint, bract
28	*M. luteus*	Leaves
35	*M. luteus*	Bract
30	*M. roseus*	Branches, lint, bract
33	*M. roseus*	Branches, leaves, bract
34	*M. roseus*	Branches, flowers, bract
36	*M. roseus*	Lint
27	"*M. sedentarius*"	Leaves
31	*M. varians*	Bract
32	*Staphylococcus hominis*	Lint
16, 26	*S. hominis*	Lint, bract

*Strain numbers on the same line indicate similar colonial morphologies. Organisms listed more than once had distinctive colony types or were isolated from different parts of the plant.

profile (see chapter 6 of this book). The morphological features used included colony characteristics (color, diameter, margin, consistency, shape, elevation), production of soluble pigments, cellular morphology (asporogenous or spore-forming rods or cocci), and staining characteristics (including gram and spore stains). Sporogenous rods were evaluated to see whether parasporal crystals were present. Asporogenous rods were characterized as pleomorphic or occurring as V-forms, in palisades, or as branching rods. Formation of a transient or permanent mycelium was determined, as well as whether they underwent a rod-coccus cycle or produced metachromatic granules. Formation of tetrads or clusters by cocci was determined. Biochemical features were determined by conventional procedures.

Fatty acid analyses were performed according to the methods described by the manufacturer of the gas chromatography system used (Hewlett-Packard Corporation); the fatty acid profile of each isolate was compared to those of species in the Hewlett-Packard (HP) database and to fatty acid profiles found in the literature. It should be noted that some of the differences in the fatty acids contained by an organism and in the quantities detected may be attributed to differences in test conditions, growth phase of the organism, the medium on which it was grown, and the temperature and time of incubation. All of these factors are known to affect fatty acid profiles of bacteria. Finally, although the data obtained for each isolate are not present here, this information can be found elsewhere in Akinwunmi (1989).

Most of the bacteria we isolated from cotton could be readily identified because their biochemical and morphological characteristics were similar to those described in *Bergey's Manual of Systematic Bacteriology* (1984–1989) and in other publications (see the many literature citations in this chapter pertaining to *Bergey's Manual* and the literature citations in "Other Sources" at the end of this chapter). However, the identifications of a few of the isolates are tentative because: (1) the results of the biochemical tests and the fatty acid profiles gave conflicting identities, or (2) there was no genus to which the organism could be assigned on the basis of the data obtained, or (3) certain features of the organism's biochemical or fatty acid profiles were different from those of similar-named organisms described in the literature.

The characteristics for identifying these gram-positive organisms are presented in tables 11–13, and they are described briefly in the text that follows. Organisms that could not be identified with certainty and those with distinctive features are noted. For convenience, they are discussed below in groups according to their cellular morphologies, including sporogenous rods, asporogenous rods, and cocci.

Sporogenous Rods

Thirteen strains of gram-positive sporogenous rods isolated from various parts of the cotton plant were identified as species of *Bacillus*. All conformed to the descriptions of the genus as outlined in *Bergey's Manual of Systematic Bacteriology* (Claus and Berkeley 1984). They were gram-positive rod-shaped bacteria that formed endospores. As has been found for various species in the genus, some were strictly aerobic while others were facultatively anaerobic. Most were motile. The colony morphology of some, but not all, of these organisms was typical of that of species of *Bacillus,* that is, they were flat, matte-surfaced, and spreading. Species identified included *B. brevis*, *B. cereus*, *B. cereus* var. mycoides, *B. circulans*, *B. laterosporus*, *B. licheniformis*, *B. pumilus*, *B. sphaericus*, and *B. subtilis*.

Typically, members of the genus *Bacillus* contain large amounts of terminally methyl-branched fatty acids, with anteiso–15:0 (a–15:0) as the major fatty acid in most species and iso–15:0 (i–15:0) as the major fatty acid in Group lA [a group of species similar to *B. cereus* as recognized by Gordon (1989)]. In Group lA isolates, i–15:0 predominates and C_{12}, C_{13}, and C_{18} fatty acids are present. The fatty acid profiles of all isolates within this group are similar both quantitatively and qualitatively and cannot be used to distinguish among the organisms in this group. Other branched chain fatty acids present in species of *Bacillus* include terminally methyl-branched iso- and anteiso-acids with 12 to 17 carbon atoms. These were the predominant ones found in all *Bacillus* species isolated from cotton

Table 11. Physiological characteristics of gram-positive sporogenous rods

Characteristic or test	*Reference organisms*[*†]								
	1	2	3	4	5	6	7	8	9
Aerobic (A) or facultative (F)	F	F	F	F	A	A	A	A/F	A
Motility	+	-							
Voges-Proskauer	+	+	-	+	+	+	+/-	-	-
Citrate utilization	+		-						
Nitrate reduction	+		+	+	-	+	+/-		-
Arginine dihydrolase				+					
Hemolysis	ß	ß							
Gelatin hydrolysis	+		+	+	+	+	+	+	
Starch hydrolysis		+	-	+	-	+	-	+	-
Casein hydrolysis	+	+	+	+	+	+	+	+	+
Acid from glucose	+		+	+	+	+	+	+	-
Acid from xylose	-		-	+	+	+	-	+	
Acid from mannitol			+					+	
Acid from arabinose	-		-	+	+	+	-	+	
Parasporal crystals	-	-							
Predominant fatty acids									
a–15:0				+	+	+	+	+	
i– 15:0	+	+		+	+	+	+	+	
16:0		+		+	+		+	+	
i– 16:0						+			
a–17:0 and/or i–17:0				+	+	+			
Other distinctive fatty acids									
C: 12	+	+							
C: 13	+	+							
C–18	+	+							
Monounsaturated acids	+								

*Identity of the reference organisms:
1. *B. cereus*
2. *B. cereus* var. mycoides
3. *B. laterosporus*
4. *B. licheniformis*
5. *B. pumilus*
6. *B. subtilis*
7. *B. brevis*
8. *B. circulans*
9. *B. sphaericus*

†A plus sign indicates the presence of a specified trait or a positive test. A minus sign indicates the opposite. ß indicates beta (clearing) hemolysis.

plants. Except for the isolate identified as *B. sphaericus* (which contained large amounts of both i–14:0 and a–15:0), either i–15:0 or a–15:0 was the predominant acid detected, and the other branched 15-carbon acid usually was present in smaller amounts.

Some isolates had unusual features. For example, within Group 1A the *B. cereus* var. mycoides isolates, unlike most species of *Bacillus,* were nonmotile. Another isolate identified as *B. cereus* formed colonies with distinctive rootlike outgrowths that spread widely and rapidly over the surface of TSA in a tangled fashion (this is typical of both *B. cereus* var. mycoides and *B. cereus* var. *anthracis*). However, because this organism was motile and ß-hemolytic, it was identified as *B. cereus* rather than *B. cereus* var. mycoides.

Table 12. Physiological characteristics of gram-positive asporogenous rods

Characteristic or test	Reference organisms*† 1	2	3	4
Aerobic (A) or facultative (F)	A	F	A	A
Motility	-	-		+\-
Catalase	+	+		+
Oxidase				-
V or Y forms or palisades	+	+		
Rod-coccus cycle	-		+	
Substrate mycelium (transitory)	-	-	+	+
Nitrate reduction	-	-	+	+
Nitrite reduction	-	-		
Indole production	-	-		
Methyl red	-			
Voges-Proskauer	-			
Citrate utilized				-
Urea hydrolysis	-	-		
Casein hydrolysis			-	
Gelatin hydrolysis		-		
Starch hydrolysis		+		
Esculin hydrolysis		+	-	
Acid from glucose	+	+	+	+
Acid from dextrin	+			
Acid from mannitol	+			
Acid from maltose	+			
Acid from adonitol			-	
Acid from arabinose			-	+
Acid from rhamnose			-	
Acid from xylose			-	+
Predominant fatty acids				
a– 15:0	+	+		+
i – 15:0	+	+		+
16:0			+	
i – 16:0	+			
a– 17:0	+	+		
Other distinctive fatty acids				
tuberculostearic (t–19:0)			+	

* Identity of the reference organisms:
1. *Clavibacter michiganense*
2. *Propionibacterium* sp.
3. *Rhodococcus rhodochrous*
4. *Oerskovia* sp.

†A plus sign indicates the presence of the specified trait or a positive test. A minus sign indicates the opposite.

The *B. subtilis* isolates exhibited variations in their ability to ferment xylose and arabinose, sugars that typically are fermented by *B. subtilis* and all the other species in Group 1B. The *B. licheniformis* isolate produced copious amounts of slime, which is typical of this species, and produced arginine dihydrolase. The *B. pumilus* isolate failed to produce acid from arabinose. However, it is known that a few strains of *B. pumilus* fail to ferment arabinose or xylose (Knight and Proom 1950). The identity of one isolate as *B. laterosporus* was based partly on the presence of a canoe-shaped body attached to the side of the spore. It should be noted that the fatty acid profile of this organism did not closely match that of *B. laterosporus* in the HP database. The *B. brevis* isolate was distinctive because it did not form acid from carbohydrates (except glucose). Although it contained the same types of fatty acids as the strains of *B. brevis* described in the literature, it had approximately twice the amount of i–15:0 reported for *B. brevis.*

Two isolates were difficult to speciate because they had some characters that are not typical of the genus. They produced pigmented colonies and formed extremely long chains of rod-shaped cells. Although they were similar biochemically to two species, *B. azotoformans* and *B. sphaericus,* their fatty acid profiles were more like that of *B. globisporus.* They were not considered to be isolates of *B. globisporus,* however, because they were unlike this species biochemically and were not facultatively anaerobic psychrophiles. Because of the conflicting identities suggested by their biochemical profiles and other features, it is possible that these two organisms, although similar to each other and to *B. sphaericus* biochemically, may be a new subgroup of this species.

The identification of one organism as *B. circulans* was based on its biochemical characteristics more than its colonial morphology. It produced a convex, gummy orange colony [whereas colonies of *B. circulans* were described as thin, transparent, spreading, and sometimes barely visible by Claus and Berkeley (1984)]. Colonial pigmentation is unusual for species of *Bacillus*; however, pigmented colonies have been reported for the *B. circulans* complex. Pigments may confer survival value to organisms in adverse habitats (Matthews and

Table 13. Physiological characteristics of the gram-positive cocci

Characteristic or test	*Reference organisms**†					
	1	2	3	4	5	6
Aerobic (A) or facultative (F)	F	A	A	A	A	A
Pigmented colonies	+/-	+	+		+	+
Cells in tetrads		+				
Oxidase					+/-	+
Catalase	+	+				
Motility		-	-			
Coagulase	-					
Nitrate reduction	+/-	-	+	-	+/-	-
Voges-Proskauer	+/-	-			+/-	-
Citrate utilized		-	+	+	-	-
Urea hydrolysis	+	+/-	+	-	-	-
Arginine dihydrolase		-		+	-	-
Hemolysis	-					
Gelatin hydrolysis		+	+	+	-	+
Starch hydrolysis		-		-	+/-	
Esculin hydrolysis		-	-	-	+/-	+
Acid from glucose		-			+	-
Acid from arabinose	-					
Growth in 7.5 percent NaCl					+	
Growth in 5 percent NaCl						-
Growth at 37 °C					+	-
Predominant fatty acids						
a– 15:0	+	+	+	+	+	+
i – 15:0	+	+	+	+	+	+
a– 17:0	+	+	+			
18:0	+					
19:0	+					
20:0	+	-	-	-	-	-
Other distinctive fatty acids						
Branched chain 19-carbon acids	+					
i – 14:0		+				
i – 16:0		+				

* Identity of the reference organisms:

1. *Staphylococcus hominis*
2. *Micrococcus luteus*
3. *M. varians*
4. *M. sedentarius*
5. *M. roseus*
6. *M. agilis.*

† A plus sign indicates the presence of a specified trait or a positive test.
A minus sign indicates the opposite.

Sistrom 1959, Turner and Jervis 1968). *B. circulans* may exist as a pigmented colonial variant on cotton where the pigment serves to protect the organism's DNA from the mutagenic effects of exposure to intense solar radiation.

Asporogenous Rods

Isolates in this group were gram-positive rod-shaped bacteria that did not form endospores. One was an actinomycete that produced chalky colonies and a brown diffusible pigment. This organism had an extensive branching mycelium with long chains of smooth-surfaced spores borne on the aerial mycelium. It was identified as a member of the white series of the genus *Streptomyces,* but species identification was not attempted.

The other nonsporing rods were similar to those described for coryneform and nocardioform bacteria in *Bergey's Manual of Systematic Bacteriology* (Jones and Collins 1984, Lechevalier 1984). Organisms that produced a substrate mycelium, however transient, were considered to be nocardioforms; otherwise they were considered to be coryneforms. The characters used to identify the organisms are presented in table 12.

Coryneforms

Members of two coryneform genera, *Clavibacter* and *Propionibacterium,* were identified among the isolates from cotton. Most corynebacteria associated with plants form pigmented colonies in shades of yellow, orange, and pink on complex media, and all of our isolates could be distinguished by either the shade or intensity of the yellow pigment of their colonies. Five isolates were considered to be *Clavibacter (=Corynebacterium) michiganense* based on their morphology and biochemical reactions, even though they did not produce acid from any carbohydrate (as has been reported for these organisms in Davis et al. 1984). It should be noted, however, that in the study by Davis et al. (1984), production of acid from carbohydrates was evaluated in a medium supplemented with yeast extract, whereas our medium did not contain yeast extract.

The major fatty acids detected in all the *C. michiganense* strains were branched chain unsaturated acids with 16 to 18 carbons, as has been reported for *Clavibacter michiganense.* The fatty acid profiles of all the isolates were remarkably similar to each other.

The other coryneform isolate was identified as a species of *Propionibacterium* because it possessed a combination of characters typical of the genus, namely the formation of a pigmented colony, cells with a typical coryneform morphology, lack of motility, catalase positive, and facultative growth (although generally thought to be composed of strict anaerobes, many members of the genus *Propionibacterium* are aerotolerant). Its biochemical profile matched the profiles described for a number of *Propionibacterium* isolates. The predominant fatty acid was a–15:0 (75 percent), which is consistent with the findings of Moss et al. (1969), where either i–15:0 or a–15:0 was the predominant fatty acid in the strains of *Propionibacterium* studied. They found the human and the environmental isolates within the genus *Propionibacterium* to be similar morphologically and biochemically and found that strains from both groups contained common fatty acids. It is difficult to speciate isolates of *Propionibacterium*, and we did not attempt to do so.

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Two of the three isolates identified as nocardioforms produced a substrate mycelium (although it was seen only in very young colonies). The third isolate did not produce a mycelium but was similar in all other respects to the two other isolates that did form such a mycelium. Aerial mycelia were not formed by any of these isolates.

One of these strains was a typical species of *Rhodococcus* and contained 10-methyloctadecanoic or tuberculostearic acid (TBSA or t–19:0). This unusual fatty acid is typical of members of this genus and is a signature acid found also in members of the genus *Mycobacterium.* However, the isolate from cotton also contained 2-OH-i–15:0 and 17:1, which were not reported in the organisms studied by Collins et al. (1982).

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The remaining two nocardioform isolates were identified tentatively as belonging to the genus *Oerskovia,* although they grew on the medium D2, which is reportedly selective for species of *Corynebacterium*. They were identified as *Oerskovia* because both contained a–15:0 as the major fatty acid and i–15:0 as the second most predominant fatty acid; these are the major fatty acids in members of the genus *Oerskovia*. Morphologically and biochemically both isolates were non-acid-fast organisms that formed yellow colonies—features that are typical of the genus *Oerskovia*. Although *Oerskovia* are reportedly nitrate positive, both isolates from cotton were nitrate negative; otherwise they were similar biochemically to members of this genus.

One of the two strains formed a branched substrate mycelium, but no aerial mycelium. The branched substrate mycelium broke up into individual rods and branched rods. This is consistent with descriptions of the genus *Oerskovia,* which are motile actinomycetes characterized by extensively branched vegetative hyphae that break up into rod-shaped elements. The other strain did not form a substrate mycelium or branched rods and was characterized by the presence of tiny gram-positive rods.

Finally, although motility was not detected in the cotton isolates, as is typical for other members of this genus, nonmotile *Oerskovia*-like organisms have been described.

Cocci

Thirteen distinctive isolates were gram-positive cocci that occurred in pairs, tetrads, or clusters and were species of *Micrococcus* or *Staphylococcus*, as described in *Bergey's Manual of Systematic Bacteriology* (Schleifer 1986). All were nonmotile, asporogenous, and catalase positive. Ten were obligately aerobic (micrococci); the remaining three were facultatively anaerobic (staphylococci). The 10 identified as micrococci either oxidized or were unable to use glucose, whereas the remaining 3 fermented glucose, as is typical for the staphylococci.

Micrococcal isolates contained the characteristic a–15:0 and i–15:0 fatty acids in the typical 2:1 ratio. They also contained i–17:0, which is a fatty acid that is not found in staphylococci. Also, as is typical for micrococci, branched chain 19-carbon acids and 18:0 and 20:0 acids were absent.

The fatty acids typical of the staphylococci were either branched chains of 15, 17, or 19 carbons or straight chain 18:0 or 20:0 acids. Descriptions of the other properties used to identify these organisms are found in table 13.

The isolates identified as species of *Micrococcus* included strains of *M. agilis, M. luteus, M. roseus*, *M. varians*, and *M. sedentarius*. All possessed the other characteristics of the genus as outlined in *Bergey's Manual of Systematic Bacteriology,* including strictly aerobic metabolism, tetrad cellular arrangement, and catalase-positive, nonmotile cocci that produce acid but not gas from glucose. Table 13 contains additional information about named micrococci described in the literature and lists the characteristics we used to identify our organisms.

The major fatty acids found in all species of *Micrococcus* are a–15:0 and i–15:0. Usually there is twice the amount or more of anteiso acid than iso acid, but there are exceptions. Unsaturated fatty acids are found as minor components, and cyclopropane acids are absent. The major acids in our isolates were a–15:0 and i–15:0; they contained no detectable cyclopropane acids and only trace to small amounts of various unsaturated fatty acids. The branched chain 19-carbon acids and 20:0 acids, all of which are typical of the staphylococci, were not

detected. The only exception was the isolate identified as *M. sedentarius*. Its fatty acid profile was not typical of the micrococci.

Five of the micrococcal isolates formed colonies that were shades of yellow. Three were identified as *M. luteus*. Two of these formed colonies that were morphologically characteristic of the species, but the other formed flat to umbonate colonies with a ring at the edge. All three contained predominantly branched chain acids with the major acids being a–15:0 and i–15:0 in approximately a 2:1 ratio.

The fourth isolate, which was identified as *M. varians*, formed pale greenish-yellow colonies that could be distinguished from those of *M. luteus* because they had a lower convex profile. Although biochemically similar to described strains of *M. varians*, this organism contained lesser amounts of both a–17:0 and i–15:0 fatty acids than reported for this species.

The fifth micrococcal isolate that formed yellow colonies was identified tentatively as *M. sedentarius*. Although it definitely was coccus and its cellular morphology did not change from 24 hr to 7 days of incubation, this isolate had a fatty acid profile more like that of a *Bacillus* than a *Micrococcus* sp. It contained i–17:0, i–17:1, and a–17:0 as its major fatty acids. These acids are either not present or not found in such large concentrations in micrococci or staphylococci. It did contain i–15:0 and a–15:0 fatty acids, however, which are the major ones reported for the genus. No information could be found in the literature or in the HP database about the fatty acid profile of *M. sedentarius*; therefore, we do not know if *M. sedentarius* has an atypical fatty acid profile.

The five remaining micrococcal isolates produced red or orange colonies of varying morphologies. Only one of these had a biochemical pattern that matched those of the red micrococci described in the literature. However, all five contained large amounts of a–15:0 and lesser amounts of i–15:0 fatty acids, typical of species of *Micrococcus.* None contained any 20:0 fatty acids. Determining whether 20:0 acids are present is important since staphylococci, like the micrococci, contain large amounts of i–15:0 and a–15:0 acids but the staphylococci also contain 20:0 acids.

Since they are so variable biochemically, it is possible to confuse micrococci that form red colonies with coryneforms, which also form red colonies or cocci at some stage of their life cycle *(Arthrobacter* or *Rhodococcus).* However coryneforms contain tuberculostearic acid and 2-hydroxy acids, neither of which was detected in any of the cotton isolates identified as a micrococcus.

Although the fatty acid profiles of the red species were helpful in determining the genus to which these isolates belonged, they were not of any help in identifying species. The profiles of only two red species, *M. roseus* and *M. agilis,* were found in the literature, and they were very similar.

One of the isolates was more similar biochemically to *M. agilis* than *M. roseus*, and it contained most of the same fatty acids in roughly the same amounts as described for strains of *M. agilis*. However, it should be noted that this isolate also contained the 16:1 acid in approximately the same amounts reported for *M. roseus* but not *M. agilis*.

The other red and orange strains of micrococci all grew at 37 °C and in the presence of 7.5 percent NaCl, as did many of the strains of *M. roseus* and *M. kristinae* tested by Kloos et al. (1974), whereas the strain identfied as *M. agilis* did not. The isolates from cotton were biochemically more similar to *M. roseus* than *M. kristinae* (an organism usually isolated from human skin), although the isolates from cotton were nitrate negative whereas 75 percent of the strains of *M. roseus* are reported to be nitrate positive. Since the fatty acid profiles of the isolates did not match the profile for *M. kristinae* in the HP database, we considered these isolates to be nitrate-negative strains of *M. roseus.*

The remaining isolates were all identified as strains of *Staphylococcus hominis.* Biochemically and by fatty acid profiles, they could have been identified as either *S. epidermidis* or *S. hominis,* but *S. hominis* was chosen because all of the staphylococci formed colonies with concentric rings, which is characteristic of *S. hominis*.

Conclusions

The taxonomic problems associated with identifying species to which *Bacillus* isolates belong and

the genus to which asporogenous rods belong are well recognized, especially if the strains are environmental and not clinical isolates. Adding the fatty acid profile as an identification criterion to morphological and biochemical characters for the *Bacillus* isolates from cotton was helpful in identifying their species.

Identifying some of the species of the staphylococci and micrococci from cotton was a problem when they were not biochemically similar to organisms described in the literature. For these isolates, information about their taxonomic features (colonial and cellular morphologies and their staining reactions) and their biochemical reactions were combined with their fatty acid profiles to establish their identities. Even so, a few of the red-pigmented micrococci fit no established pattern. However, the lack of 10-tuberculostearic acid did allow recognition of these isolates as micrococci rather than coryneforms.

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Asporogenous Rods

Isolates in this group were gram-positive rod-shaped bacteria that did not form endospores. One was an actinomycete that produced chalky colonies and a brown diffusible pigment. This organism had an extensive branching mycelium with long chains of smooth-surfaced spores borne on the aerial mycelium. It was identified as a member of the white series of the genus *Streptomyces,* but species identification was not attempted.

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The fourth isolate, which was identified as *M. varians*, formed pale greenish-yellow colonies that could be distinguished from those of *M. luteus* because they had a lower convex profile. Although biochemically similar to described strains of *M. varians*, this organism contained lesser amounts of both a–17:0 and i–15:0 fatty acids than reported for this species.

The fifth micrococcal isolate that formed yellow colonies was identified tentatively as *M. sedentarius*. Although it definitely was coccus and its cellular morphology did not change from 24 hr to 7 days of incubation, this isolate had a fatty acid profile more like that of a *Bacillus* than a *Micrococcus* sp. It contained i–17:0, i–17:1, and a–17:0 as its major fatty acids. These acids are either not present or not found in such large concentrations in micrococci or staphylococci. It did contain i–15:0 and a–15:0 fatty acids, however, which are the major ones reported for the genus. No information could be found in the literature or in the HP database about the fatty acid profile of *M. sedentarius*; therefore, we do not know if *M. sedentarius* has an atypical fatty acid profile.

The five remaining micrococcal isolates produced red or orange colonies of varying morphologies. Only one of these had a biochemical pattern that matched those of the red micrococci described in the literature. However, all five contained large amounts of a–15:0 and lesser amounts of i–15:0 fatty acids, typical of species of *Micrococcus*. None contained any 20:0 fatty acids. Determining whether 20:0 acids are present is important since staphylococci, like the micrococci, contain large amounts of i–15:0 and a–15:0 acids but the staphylococci also contain 20:0 acids.

Since they are so variable biochemically, it is possible to confuse micrococci that form red colonies with coryneforms, which also form red colonies or cocci at some stage of their life cycle *(Arthrobacter* or *Rhodococcus)*. However coryneforms contain tuberculostearic acid and 2-hydroxy acids, neither of which was detected in any of the cotton isolates identified as a micrococcus.

Although the fatty acid profiles of the red species were helpful in determining the genus to which these isolates belonged, they were not of any help in identifying species. The profiles of only two red species, *M. roseus* and *M. agilis,* were found in the literature, and they were very similar.

One of the isolates was more similar biochemically to *M. agilis* than *M. roseus*, and it contained most of the same fatty acids in roughly the same amounts as described for strains of *M. agilis*. However, it should be noted that this isolate also contained the 16:1 acid in approximately the same amounts reported for *M. roseus* but not *M. agilis*.

The other red and orange strains of micrococci all grew at 37 °C and in the presence of 7.5 percent NaCl, as did many of the strains of *M. roseus* and *M. kristinae* tested by Kloos et al. (1974), whereas the strain identfied as *M. agilis* did not. The isolates from cotton were biochemically more similar to *M. roseus* than *M. kristinae* (an organism usually isolated from human skin), although the isolates from cotton were nitrate negative whereas 75 percent of the strains of *M. roseus* are reported to be nitrate positive. Since the fatty acid profiles of the isolates did not match the profile for *M. kristinae* in the HP database, we considered these isolates to be nitrate-negative strains of *M. roseus*.

The remaining isolates were all identified as strains of *Staphylococcus hominis*. Biochemically and by fatty acid profiles, they could have been identified as either *S. epidermidis* or *S. hominis,* but *S. hominis* was chosen because all of the staphylococci formed colonies with concentric rings, which is characteristic of *S. hominis*.

Conclusions

The taxonomic problems associated with identifying species to which *Bacillus* isolates belong and

the genus to which asporogenous rods belong are well recognized, especially if the strains are environmental and not clinical isolates. Adding the fatty acid profile as an identification criterion to morphological and biochemical characters for the *Bacillus* isolates from cotton was helpful in identifying their species.

Identifying some of the species of the staphylococci and micrococci from cotton was a problem when they were not biochemically similar to organisms described in the literature. For these isolates, information about their taxonomic features (colonial and cellular morphologies and their staining reactions) and their biochemical reactions were combined with their fatty acid profiles to establish their identities. Even so, a few of the red-pigmented micrococci fit no established pattern. However, the lack of 10-tuberculostearic acid did allow recognition of these isolates as micrococci rather than coryneforms.

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Chapter 5. The Gram-Negative Bacterial Flora of Cotton

0.0. Akinwunmi and C.E. Heintz

Cotton grown in west Texas is contaminated with large numbers of gram-negative bacteria (GNB), on the order of 10^6 to 10^8 per gram of dry weight of fiber (Morey et al. 1983, Heintz et al. 1988 and 1989). The outer cell wall layer of these organisms contains lipopolysaccharides referred to as endotoxin. When cotton is processed, dusts having a high concentration of GNB and endotoxin are produced. Inhalation of the dust has been implicated as the cause of byssinosis, a debilitating respiratory disease of workers in cotton mills (see chapter 1 in this book and the various Proceedings of the Cotton Dust Research Conferences).

Lint within unopened bolls is sterile (Rylander and Lundholm 1978, Rylander 1981, Simpson et al. 1983, DeLucca and Palmgren 1986) but rapidly becomes contaminated with GNB after the bolls open (Heintz et al. 1988). Moisture plays a major role in the development of large numbers of GNB on the lint. If the fiber gets wet after the bolls open, high levels of these organisms can be isolated subsequently from the lint (DeLucca et al. 1990, Heintz et al. 1990, Batson et al. 1991, Zuberer and Kenerley 1991, Fischer and Foarde 1992).

Although considerable information is available on the predominant GNB found on lint (Rylander and Lundholm 1978, Fischer and Kylberg 1983, DeLucca and Palmgren 1986, Simpson et al. 1988 and 1989), little is known about the types and numbers of GNB found on other parts of the cotton plant. Therefore, we undertook a project to identify the heterotrophic gram negative flora that could be isolated under mesophilic, aerobic conditions. All the major parts of the plant, including the lint, were examined throughout the growing season in 1986. Each different colonial type was included in this study, but no attempt was made to quantitate specific types of organisms. In 1987, after we had learned how to distinguish among these bacteria, the numbers of dominant types of organisms on each cotton plant part were determined (Akinwunmi 1989, Akinwunmi et al. 1989).

From the 1987 study results, we hoped to determine whether the predominant gram-negative lint colonizers (for example, *Enterobacter agglomerans*, *Pseudomonas syringae*, and *Flavimonas oryzihabitans*) were present on other parts of the cotton plant prior to boll opening. If so, then the cotton plant itself could be the source of the bacterial inoculum for the organisms that colonize or contaminate the lint. Knowing how, when, and why the predominant GNB appear on the lint might suggest an intervention strategy to prevent them from becoming established. One possibility is to try to replace these organisms with other components of the normal flora. Field studies performed by Fischer et al. (1989) showed that gram-negative organisms could be displaced by spraying the lint with gram-positive organisms. Reducing the number of GNB on the lint (and consequently the amount of endotoxin) could lead to the production of cotton that liberates fewer organisms when carded in the mills.

Study Procedures

The1986 study to isolate and identify the gram-negative bacterial flora from different parts of the cotton plant used Paymaster 404 variety plants grown at an agricultural experiment station in Lubbock, TX. The procedures were the same as those used in a companion study on gram-positive bacteria (see chapter 4). Table 14 identifies the parts of the cotton plant on which each strain was found during the growing season.

All isolates were either glucose-fermenting or glucose-nonfermenting rods. Information from the literature used in the identification of these organisms is found in tables 15 and 16. The original data for each of our isolates is found in Akinwunmi (1989). Identifications were based on a variety of phenotypic information, including cellular and colonial morphologies, staining reactions, results of biochemical tests, and fatty acid profiles. The biochemical tests included the IMViC series (that is, indole, methyl red, Voges-Proskauer, and citrate tests), tests for cytochrome oxidase activity, nitrate and nitrite reduction, starch, urea, gelatin, and esculin hydrolysis, and tests to determine whether each organism could produce lysine or ornithine decarboxylases, arginine dihydrolase, or hydrogen

Table 14. GNB and the plant parts on which they were found

Strain numbers	Species*	Plant parts
Enterobacteriaceae		
51	*Enterobacter agglomerans*	All plant parts except seeds
53	*E. agglomerans*	All plant parts, including seeds
54	*E. agglomerans*	All plant parts, including seeds
60	*E. agglomerans*	Roots, stems, branches, leaves, and lint
49	*E. agglomerans* (*Escherichia adecarboxylata*?)	Lint
41	*Enterobacter cloacae*	Roots, lint
48	*E. cloacae*	Roots
55	*E. cloacae*	Roots
58	*Serratia liquefaciens*	Roots
Pseudomonadaceae		
44	*Pseudomonas syringae*	All plant parts except bracts and seeds
46	*P. syringae*	All plant parts except flowers and seeds
50	*P. syringae*	All plant parts except seeds
52	*P. syringae*	All plant parts except seeds
42	*Flavimonas oryzihabitans*	All plant parts except flowers and seeds
45	*F. oryzihabitans*	All plant parts except seeds
40	*Pseudomonas fluorescens/ P. putida*	Roots, lint
57	*Pseudomonas fluorescens/ P. putida*	Roots, lint
38	*P. solanacearum*	Stems
Rhizobiaceae		
37	*Agrobacterium radiobacter*	Roots
56	*Rhizobium* sp.	Roots
Neisseriaceae		
59	*Acinetobacter calcoaceticus* biovar *Lwoffi*	Roots, leaves, lint, and bracts

*Species listed more than once formed distinctive colonies that were isolated from different parts of the plant.

sulfide. The formation of acid from glucose, xylose, and mannitol and the production of gas from glucose was evaluated. Whether each isolate was aerobic or facultative and motile or nonmotile was determined.

Glucose-Fermenting Rods

Nine morphologically distinct colonies isolated from cotton plant parts were identified as glucose-fermenting gram-negative rods. They were facultatively anaerobic, oxidase negative, catalase positive, and motile by peritrichous flagella and therefore conformed to the description of members of the family Enterobacteriaceae as outlined in *Bergey's Manual of Systematic Bacteriology* (Brenner 1984). All members of the family Enterobacteriaceae have similar fatty acid profiles. Typically 16:0 acid is the most abundant acid, followed by the 16:1, 18:1, and

Table 15. Physiological characteristics of gram-negative fermenters (Enterobacteriaceae)

Characteristic or test	Reference organisms*†			
	1	2	3	4
Aerobic (A) or facultative (F)	F	F	F	F
Motility	+	+	+	+
Indole	-	-	+	+
Methyl red	-	-	-	+
Voges-Proskauer	+	+	+	-
Citrate	+	+	+	-
Nitrate reduction	+/-	+	+	
Nitrite reduction	-	-		
Urea hydrolysis		+/-	-	
Gelatin hydrolysis	-	-		
Lysine decarboxylase	-	-	+	-
Arginine dihydrolase	-	+	-	-
Ornithine decarboxylase	-	+	+	-
Hydrogen sulfide produced	-	-	-	-
Acid from glucose	+	+	+	+
Acid from xylose	+	+	+	
Acid from mannitol	+	+	+	
Gas from glucose	+/-	+	+	+
Esculin hydrolysis				+
Predominant fatty acids				
16:1	+		+	+
16:0	+	+	+	+
18:1	+		+	+
17:0 cyc		+		+
19:0 cyc				+
Other distinctive fatty acids				
Hydroxy acids			+	

*Identity of the reference organisms:

1. *Enterobacter agglomerans*
2. *E. cloacae*
3. *Serratia liquefaciens*
4. *Escherichia adecarboxylata* and *E. coli.*

† A plus sign indicates the presence of the specified trait or a positive test.
A minus sign indicates the opposite.

Table 16. Physiological characteristics of gram-negative nonfermenters

Characteristic or test	Reference organisms*†								
	1	2	3	4	5	6	7	8	9
Aerobic (A) or facultative (F)	A	A	A	A	A	A	A	A	A
Motility	+	+	+	+	+	+	+	+	-
Type of flagellation‡	P	P	P	P	P	P	P/PE	PE	-
Fluorescent pigment produced	-	+	+	-	-	+			
Oxidase reaction	-	+	+	-	+	-		+	-
Urea hydrolysis	+/-							+	
Starch hydrolysis		-						-	
Gelatin hydrolysis		+	-					-	
Esculin hydrolysis	-							+	
Nitrate reduction	-	+	-	+	+	-		+	-
Nitrite reduction	-	+	-		+	-			-
Lysine decarboxylase	-							-	-
Arginine dihydrolase	-	+	+		-	-			-
Ornithine decarboxylase	-							-	-
Predominant fatty acids									
16:1	+	+	+			+			
16:0	+	+	+	+	+	+			+
18:1	+	+			+	+	+	+	+
Other distinctive fatty acids									
Hydroxy acids	+	+	+	+	+	+		+	
Iso-branched acids				+					

*Identity of the reference organisms:

1. *Flavimonas oryzihabitans*
2. *Pseudomonas fluorescens*
3. *P. putida*
4. *P. maltophilia*
5. *P. solanacearum*
6. *P. syringae*
7. *Rhizobium* sp.
8. *Agrobacterium radiobacter*
9. *Acinetobacter calcoaceticus* biovar. *Lwoffi.*

†A plus sign indicates the presence of the specified trait or a positive test; a minus sign indicates the opposite.

‡*P*, Polar. *PE*, Peritrichous.

17:0 cyc acids. Except where noted in the text that follows, all of the organisms were biochemically similar to named organisms and had fatty acid profiles typical of the species (see characteristics of named organisms in table 15).

All of the glucose fermenters were species of either *Enterobacter* or *Serratia.* Five were considered to be biovars of *E. agglomerans.* Four of these were identified as anaerogenic strains of *E. agglomerans* because they did not produce gas from glucose fermentation. Three of these produced yellow-pigmented colonies, and the fourth was nonpigmented. Although most species of this organism form pigmented colonies, nonpigmented forms of *E. agglomerans* are known.

The remaining *E. agglomerans* isolate was quite different from the others. It was more similar to *Escherichia adecarboxylata* than to *E. agglomerans* because of its lack of colony pigmentation. Also, both its biochemical and fatty acid profiles were more like those of *Escherichia* sp. However, biochemically and by the fatty acids it contained, it also fit the descriptions of the aerogenic, nonpigmented organisms assigned to biogroup G3 of *Enterobacter aerogenes.* Since it did not contain the fatty acid 19:0 cyc, an acid usually detected in species of *Escherichia,* this organism was tentatively identified as an aerogenic species of *Enterobacter agglomerans.* It has been suggested that *E. adecarboxylata* is closely related to, and belongs in, the *Erwinia-E. agglomerans* complex.

Three organisms were typical isolates of *Enterobacter cloacae* except that they contained fewer 16:0 and 17:0 cyc acids than described for some isolates of *E. cloacae.* The fatty acid database that we used matched the profile of one of these organisms (that of *Erwinia chrysanthemi*); identified another as either *Salmonella cholerasuis, S. typhimurium, S. typhi, S. enteritidis,* or *Escherichia coli;* and indicated that the remaining isolate was *Kluyvera cryocrescens.* The biochemical profiles of these isolates indicated such identifications were not likely and that these organisms were isolates of *E. cloacae.* The caveat is that environmental isolates are not always similar to clinical isolates of the same organism, and more information may be needed to properly identify bacteria isolated from nonclinical sources. This also was noted for the final glucose-fermenting isolate described below.

The remaining isolate was identified as *Serratia liquefaciens* (previously *Enterobacter liquefaciens)* because its biochemical reactions were typical of this species, although it did not match the profile of *Serratia liquefaciens* in the fatty acid database, which listed *Klebsiella terrigena, E. chrysanthemi,* and *E. herbicola,* as possible matches. This organism did not resemble any of these species biochemically, however.

Glucose-Nonfermenting Rods

Thirteen organisms were identified as glucose-nonfermenting gram-negative aerobic rods. They were classified in the families Pseudomonadaceae, Rhizobiaceae, and Neisseriaceae. For convenience, the isolates are described by family. Characteristics of the named organisms used to identify these isolates are listed in table 16.

Family Pseudomonadaceae

Ten isolates were placed in the family Pseudomonadaceae. This group included one organism, *Xanthomonas maltophilia*, that previously was classified in the genus *Pseudomonas* as *P. maltophilia*. The others were identified as *Flavimonas oryzihabitans* (formerly *Pseudomonas* sp. Ve–2), *P. fluorescens*, *P. putida*, *P. solanacearum*, and *P. syringae*. All of these isolates conformed to the description of members of the family Pseudomonadaceae as outlined in *Bergey's Manual of Systematic Bacteriology* (Palleroni 1984). They were strictly aerobic, nonfermentative, catalase positive, oxidase positive or negative, and motile by means of polar flagella. All contained large amounts of 16:0, 16:1, and 18:1 fatty acids and hydroxylated fatty acids typical of the genus *Pseudomonas.*

The isolate that was identified as *X. maltophilia* contained branched hydroxy acids and isobranched acids that are found in several species of *Xanthomonas,* including *X. maltophilia,* but not in species of *Pseudomonas.* However, this isolate was nonpigmented and reduced nitrates to nitrites—two traits that are not typical of the species *Xanthomonas.*

Two of the isolates were oxidase-negative and motile by a single polar flagellum and produced yellow, nonfluorescent, nondiffusible colonial pigments. These two isolates were identified as members of the CDC Ve–2 group of *Pseudomonas,* now named *Flavimonas oryzihabitans.* They were not considered to be *X. maltophilia* (which is the only other yellow-pigmented, oxidase-negative pseudomonad with a single polar flagellum) because they lacked the branched hydroxy- and iso-acids typical of *X. maltophilia.*

Two of the fluorescent isolates had biochemical characteristics and fatty acid profiles common to both *P. fluorescens* and *P. putida.* The biochemical test results indicated they were either denitrifying

strains of *P. putida* (although none have been reported in the literature) or gelatinase-negative strains of *P. fluorescens* biovar II. Since the fatty acid profiles of *P. fluorescens* and *P. putida* are similar, a choice between the two species could not be made.

The remaining four fluorescent pseudomonads were identified as *P. syringae* on the basis of their biochemical profiles. Also, their fatty acids were similar to those of *P. syringae* reference organisms listed in the database that we used.

The remaining isolate was identified as *P. solanacearum* because it produced a nonfluorescent yellowish colony and copious amounts of a brown diffusible pigment—traits characteristic of *P. solanacearum.* This isolate also had a biochemical profile and growth temperature response similar to those described in the literature for *P. solanacearum.* It contained the same fatty acids in roughly the same amounts as those described for this species, except that the 18:1 acid was found in higher concentration in the isolate from cotton.

Family Rhizobiaceae

Two gram-negative glucose-nonfermenting rods conformed to the description of members of the family Rhizobiaceae as outlined in *Bergey's Manual of Systematic Bacteriology* (Jordan 1984). They were aerobic rods, motile by one polar or subpolar flagellum or two to six peritrichous flagella, and they produced considerable extracellular slime during growth on media containing carbohydrate. The fatty acids of the species in this family that have been examined so far are dominated by cis-18:1 and the derived cyclopropane acid, 19:0 cyc.

One of these organisms biochemically was like *Agrobacterium radiobacter.* It produced glistening, olive green colonies on a D1 medium, which is selective for nitrate-using strains of *Agrobacterium,* and the isolate produced one to four peritrichous flagella after 18 hr in culture. Further, it grew rapidly on media containing carbohydrate. Although the isolate contained characteristic 3–OH–14:0, 3–OH–16:0, and 18:1 acids, it also had a large amount of the 19:0 cyc acid (20 percent). This was considered to be an artifact of aging, since this organism had to be grown for 72 hr before sufficient cell mass was available for testing. In several GNB, including members of the genera *Escherichia, Serratia, Agrobacterium, Pseudomonas,* and *Yersinia,* it is known that the 18:1 acid is converted to 19:0 cyc as the cells age.

The other isolate in this family was identified as a species of *Rhizobium* because it was motile by means of 1 to 2 polar flagella after 18 hr in culture, it grew rapidly on yeast mannitol agar, and produced colonies larger than 1 mm after 3 days of incubation. Its fatty acid profile was consistent with that of the genus except that considerably larger amounts of the 18:1 acid (78 percent) were detected.

Family Neisseriaceae

One isolate was placed in the family Neisseriaceae. It conformed to the description of the members of the family as outlined in *Bergey's Manual of Systematic Bacteriology* (Bøvre 1984). Members of the family Neisseraceae occur as singles or pairs of cocci, or as masses of cocci, often with adjacent sides flattened. The cells have a tendency to resist decolorization after staining, and they lack flagella. All species are aerobic.

Acinetobacter calcoaceticus is the only oxidase-negative member of the family Neisseriaceae and does not grow on MacConkey agar. Although the only recognized species is *A. calcoaceticus*, several distinct phenotypic groups are recognized. Distinctions among them include whether they produce acid from glucose oxidatively and whether they are hemolytic. Our isolate did not grow on MacConkey agar; it was nonmotile, nonhemolytic, and oxidase negative. It was negative for all biochemical reactions except that it did hydrolyze urea. Based on these results, the organism was identified as *Acinetobacter calcoaceticus* biovar *Lwoffi.* A usable fatty acid profile was not obtained.

Conclusions

Although *E. agglomerans* and *P. syringae* have been shown by others to be two of the most significant organisms on lint in terms of numbers (and therefore presumably in the amount of endotoxin produced), there are a number of other types of gram-negative organisms associated with cotton that

presumably contribute to the endotoxin load and therefore to the potential of respiratory disease in exposed individuals. Both glucose-fermenting and glucose-nonfermenting gram-negative rods in the families Enterobacteriaceae, Pseudomonadaceae, Rhizobiaceae, and Neisseriaceae were isolated from various parts of the cotton plant and identified in this study. Both here and elsewhere (Akinwunmi et al. 1989, Grice and Heintz 1990), GNB were reported to be found everywhere on the plant, including the seeds enclosed in the bolls. However, each kind was not constantly present on all parts of the cotton plant.

In addition, the predominant GNB (those most often found on each plant part) and the most abundant ones (those found in highest numbers) varied from part to part and were detected at different times during the growing season (Akinwunmi 1989). The gram-negative organisms commonly found on lint, including *E. agglomerans, P. syringae,* and now *F. oryzihabitans,* were present in large numbers on other plant parts prior to boll opening. Thus the plant itself is a likely source of the bacterial inoculum that contaminates the lint, especially since the transfer of organisms from leaves (or other parts) to opening bolls is readily accomplished as the plants rub against each other in the wind or as splashing occurs from droplets in rain or heavy dew. Since the sterile (or relatively uncolonized) lint within unopened bolls acquires its bacterial flora shortly after the bolls open, treatment of the entire plant to reduce its gram-negative flora prior to opening of the first bolls may reduce the number of these bacteria and thus the amount of endotoxin that ultimately is found on the lint.

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Chapter 6. Methods for Identifying Viable Organisms Associated with Cotton, the Cotton Plant, and Cotton Dust[1]

Caryl E. Heintz

Some organisms on cotton, in cotton dust, or in the air of textile mills are difficult to isolate and identify with certainty once isolated (Heintz 1992). In addition the water-, saline-, or buffer-extractable bacterial flora may differ both quantitatively and qualitatively depending on where the cotton is grown, environmental conditions during growth and storage, and the length of time the cotton is stored. In spite of these variables, certain organisms are commonly isolated. These are the ones that occur most frequently (the predominant organisms) and the ones that are found in the greatest number (the most abundant organisms).

This chapter provides information from studies performed by several researchers[1] working to develop procedures for isolating and identifying bacteria found on cotton. Some procedures and results are given, and some advice is summarized. The studies focused on the following:

1. The kinds of flora isolated (recoverable) and the kinds missed (nonrecoverable) using different plating and incubation techniques
2. The most commonly found organisms and those present in the highest numbers
3. The criteria used to identify the organisms and the references and databases used to determine which criteria and tests should be used for identification
4. Some known pitfalls regarding interpreting the data and identifying the organisms
5. The number of tests that should be done to identify organisms and which tests should be used when dealing with large numbers of organisms or with just a few organisms (to be as certain as possible of their identities)
6. Reasons for characterizing organisms thoroughly and situations where a superficial characterization is more appropriate.

[1] This chapter is a summary of Fischer et al. (1992) and Heintz (1992).

Isolation of Bacteria

The general types of organisms that grow on full-strength primary-plating media (nutrient agar [NA] or tryptic soy agar [TSA]) are heterotrophic bacteria that grow at mesophilic incubation temperatures (25 to 37 °C); thermophiles can be recovered if the incubation is 45 to 60 °C.

If plating and incubation procedures are used for the general types of organisms, anaerobes, psychrophiles, autotrophs, bacteria that grow only on dilute media, slow-growing organisms (such as those that require more than 10 days to form colonies at mesophilic temperatures), and specialized physiologic types such as nitrogen-fixing organisms will not be recovered.

The most prevalent and most abundant organisms isolated from cotton using heterotrophic isolation media and mesophilic incubation temperatures were as follows:

Type of Bacteria	Species
Gram-positive rods	*Bacillus* sp.
	Clavibacter michiganense
Gram-positive cocci	*Micrococcus luteus*
	M. roseus
Gram-negative fermentative rods	*Enterobacter agglomerans*
	E. cloacae
Gram-negative nonfermentative rods	*Pseudomonas syringae* *P. fluorescens*
	Flavimonas oryzihabitans
	Xanthomonas maltophilia
	Protomonas extorquens (sporadic)

Criteria Used To Identify Bacteria

The criteria used to identify these bacteria included their ability to grow on selective media, colony morphology, pigmentation of the colony, ability to

produce soluble pigments (such as fluorescent pigments), cellular morphology and arrangement, gram stain results, spore stain results, biochemical profile, and fatty acid profile.

Biochemical profiles were determined by either using the API rapid test systems (Analytab Products Incorporated) or by using conventional-tubed media. When the API systems were used, "profile numbers" generated by the data were matched with those in the API database to help identify the organism. However, this manufacturer's databases include more clinical than environmental isolates, and at times, there was a low probability of a correct identification.

Fatty acid profiles were determined by conventional methods using reference data from the Hewlett-Packard microbial identification system database and from the published literature.

Based on their colonial and cellular morphology and gram staining reactions, the organisms were divided into four groups. The most useful tests or characteristics for identifying organisms in each of the morphologically defined groups were as follows:

Gram-Positive Sporogenous Rods

- Presence of spores (spore or methylene blue stain)
- Citrate utilization
- Voges-Proskauer and nitrate reduction tests

Gram-Positive Coryneforms

- Catalase positive
- "Chinese letter" or "V" form cellular arrangements
- Lack blood hemolysis
- Generally inert biochemically
- Some use citrate

Gram-Positive Cocci

- Aerobic or facultatively anaerobic
- Oxidize or ferment glucose
- Cellular morphology is as tetrads or clusters

Gram-Negative Fermentative Rods

- Glucose is fermented anaerobically
- API 20E (or traits determined from conventional tests such as those listed below)
- Oxidase negative
- Produces gas from glucose
- IMViC (indole, methyl red, Voges-Proskauer, citrate) tests
- Decarboxylase reactions (lysine, ornithine, arginine)
- Fluorescence negative

Gram-Negative Nonfermentative Rods

- Do not ferment glucose
- Glucose is oxidized or sugars are not used (nonsaccharolytic)
- Produce soluble pigments (+ fluorescence)
- Motile or nonmotile
- Antisera to known flagellar types
- API 20NFT (or traits determined from conventional tests such as those listed below)
- Oxidase positive or negative
- Grow on a soluble starch (SX) medium for *Xanthomonas* sp.
- Nitrate is reduced.

Known Pitfalls in Identifying Isolates

Several problems pertaining to data interpretation and identifying organisms occurred in the studies. In response to these problems, the researchers gave the following warnings and advice:

1. Databases compiled using results obtained from clinical isolates can lead to mistakes in the identification of environmental isolates.
2. Verify results when new rapid identification systems and databases are used.
3. Organisms' names may change as databases are upgraded and bacteria are reclassified.
4. Biotypes vary and the same organism may have different phenotypes.

5. Commonly encountered organisms (such as *Enterobacter aerogenes* and *Pseudomonas syringae)* may have more than one type of colony. If this is not recognized, the flora may be considered more diverse than it actually is.
6. If the lecithinase reaction is used to help identify species of *Bacillus,* some isolates produce a false-positive reaction ("footprint") under the colony. The reaction may spread beyond the edge of the colony on incubation.
7. If the colonies of gram-positive organisms are allowed to remain at room temperature, they will lose their typical gram-staining reaction and will appear to be gram-negative. This is especially true for *Bacillus* sp.
8. Since some *Bacillus* sp. lose their gram-positive staining reaction, it is advisable to do spore stains before doing gram stains so that these organisms can be recognized.
9. Unless the morphology and the gram-staining reaction of the cells from young colonies is known, an organism may be inoculated into the wrong kind of rapid identification system, and it will be misidentified (for instance, a *Bacillus* can be identified as an *Enterobacter* using API strips).
10. *Pseudomonas syringae* will not be identified properly on an API–NFT strip. Because it produces a fluorescent pigment, it will be misidentified as *Pseudomonas fluorescens.*
11. An organism's biochemical profile may indicate that it should be identified one way, while its fatty acid profile may indicate that it should be identified as another organism. Conflicts usually can be resolved by doing more biochemical tests or by using selective or differential media that will rule out one or more of the choices.
12. There may be some organisms that cannot be identified because they have not been described previously in the literature.

Selection of Tests for Identification of Isolates

Routinely, the following information is used to identify isolates of interest:

- Colony morphology and pigmentation
- Presence of soluble pigments
- Gram stain (or KOH gram stain)
- Cellular morphology and arrangement
- Spore stains for gram-positive bacilli
- OF glucose for gram-negative rods
- Oxidase test for gram-negative rods.

When organisms have to be characterized more completely or when it is necessary to establish a positive identification, a biochemical profile using tests appropriate to the type of organism being identified is needed. In addition an organism's fatty acid profile can be used to help identify an organism or to confirm its identity. Other tests relying on comparisons of genetic profiles may also be useful but were not employed in these studies.

Surveying Many Isolates

If many isolates are to be characterized at one time (such as when surveys are done) or if a lot of samples must be evaluated simultaneously, the "patch plate" method used by microbial geneticists is useful. In this procedure colonies from the primary isolation medium are subcultured to known positions on fresh plates of media by referring to a numbered grid pattern placed beneath the petri dishes. All plates are inoculated in a known orientation so that colonies of each organism develop in the same position on each plate. The reactions of large numbers of isolates can be determined in this manner when each "patch" plate contains a different medium. One should be aware, however, that this approach can be foiled if one or more of the organisms is extremely motile. Motile organisms can spread rapidly over the plate, making it difficult or impossible to read the reactions of the other organisms. If this happens, repeat the procedure and subculture swarming organisms onto separate plates.

Inoculating three "patch" plates and one tube of OF-glucose medium from colonies developing on a primary isolation medium is a relatively quick and inexpensive way to obtain information useful for characterizing large numbers of bacterial isolates

from cotton. After an incubation period of 7 to 10 days occurs at room temperature, one plate is used for colonial morphology and pigmentation studies. A second plate is used as inoculum for biochemical and other tests, if necessary, and then colonies are tested for their reaction to the "KOH" gram stain. The third plate is used for the oxidase reaction (if needed). These "patch" plates contain a maintenance medium such as TSA or a differential medium such as King's B or *Pseudomonas* F for fluorescence or SX medium for *Xanthomonas* sp. Simultaneously inoculating one tube of OF-glucose broth for aerobic incubation provides information about each isolate's ability to either ferment or oxidize sugars, or to determine if it is nonsaccharolytic. Most of the predominant and abundant organisms isolated from cotton lint can be identified, at least tentatively, using this simple suite of tests.

Thorough Characterization of Isolates

A different approach is recommended when only one or a few isolates need to be identified. A complete characterization of each isolate is appropriate if the organisms are going to be used as a source of lipopolysaccharide cell wall material in other experiments or if the isolate is to be used as a reference organism or will be exchanged among laboratories. A complete characterization requires the researcher to perform all routine characterization tests (morphology, staining, etc.) and a complete biochemical characterization (API or conventional media). If possible, a fatty acid profile and genetic homology testing should be performed.

Finally, the researchers in these studies issued a plea that all data gathered in characterizing an organism be made available for exchange among laboratories.

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Chapter 7. Changes in Viable Bacterial Counts During Storage of Cotton Samples

Janet J. Fischer

Types of Cotton Samples Collected for Bacterial Counts

Researchers have performed bacterial counts on the following types of cotton plant and lint samples in the past:

1. Parts of the cotton plant obtained at different times in the growing season and analyzed as promptly as possible
2. Parts of the cotton plant obtained in the field, dried, and stored for future studies. Often these are bracts.
3. Cotton lint obtained in the field and studied promptly. The lint has been collected from bolls that vary in their stage of development or condition, including those that are unopened, damaged (usually by insects), just opened (fully fluffed), weathered in the field for a specified number of weeks, or harvested after defoliation of plant by frost or application of chemicals.
4. Seed cotton obtained from storage modules or from gins
5. Cotton lint obtained just after ginning
6. Baled cotton obtained in the mills as the bale is opened for carding, in classing offices prior to selling the bale (called Classers' samples, they are evaluated according to agreed-upon standards), or during storage after various storage periods
7. Cotton samples from other stages of processing in the mill (for example, carding, drawing,or roving)
8. Cotton dust from various sources.

Since bales of cotton and some of the cotton products listed above are stored prior to study, it is important to examine what happens to the microbiological flora during storage. This chapter focuses on this topic. Cotton samples were taken by several researchers. The studies were performed in the laboratory of Janet J. Fischer, except for the results in table 22 (which also support our data).

Bacterial Counts During Short-Term Storage of Baled Cotton

Counts of viable gram-negative bacteria (GNB) were reduced by 50 percent when bales of cotton were stored for 3 mo in 1981, and a further reduction occurred after the bales were stored for 6 mo (table 17). T tests (paired two samples for means) indicate that the harvest data (time zero) for both sets of specimens of the 1981 crop are significantly different from the 3-mo or 6-mo data, but the 3-mo data are not significantly different from the 6-mo data. The 1982 crop data in table 17 compare GNB counts on cotton harvested wet and dry. Only the data from the dry harvest (followed by ambient drying) can be compared to the 1981 data. Cotton

Table 17. Effects of storage time of baled cotton (DES–56, minimum cleaning*) on levels of GNB

Crop and conditions	GNB count (in millions cfu† /g) after: Harvest	3 Mo of storage	6 Mo of storage
1981 Crop			
Ambient drying	5.5	2.7	2.5
Dried at 250 °F	1.6	0.5	0.4
1982 Crop			
Wet harvest/ambient drying	6.7	6.1	5.1
Dry harvest/ambient drying	6.2	6.3	3.8
Wet harvest/dried at 200 °F	5.7	11.0	4.6

**DES–56*, A specific strain from the Delta Experiment Station. Minimum cleaning involved the use of dryers, a feeder, and a ginstand only.

†*cfu*, Colony forming units.

Source: Columbus and Fischer (1985).

Table 18. GNB counts (and standard deviations) from baled cotton after harvest and after 3 mo of storage

Cottons	GNB (millions cfu*/g)	
	After harvest	After storage
No. 1	2.0 ± 1.1	0.8 ± 0.2
No. 2	1.6 ± 0.9	0.5 ± 0.3
No. 3	0.5 ± 0.5	0.2 ± 0.2

**cfu*, Colony forming units.

Source: Columbus and Fischer (1985).

Table 19. Levels of GNB and GPB during long-term storage of cotton at room temperature (1980 Classers' samples)

Months of storage	Bacterial levels (in log cfu*/g)			
	Cotton No. 1		Cotton No. 2	
	GNB	GPB	GNB	GPB
**0	8.2	TNTC†	8.4	6.7
**8	6.5	--	7.0	--
12	6.6	--	6.4	5.2
19	7.5	5.8	6.9	5.2
24	6.6	6.8	6.4	6.6
30	6.5	6.7	5.6	5.3
36	6.0	5.6	5.0	5.6
42	6.0	4.5	4.8	4.6
55	4.0	5.2	4.9	5.5

**cfu*, Colony forming units.

†*TNTC*, Too numerous to count.

harvested dry shows no significant differences in the counts of GNB from harvest through 6 mo of storage. Data from cotton harvested wet are probably not reliable. Note that in one cotton harvested while wet (but dried at 200 °F) the GNB count at 3 mo actually increased, and in another cotton (also harvested wet but dried at ambient air temperatures) the count decreased but not substantially until after 6 mo of storage. The variability in counts from different cottons stored for different lengths of time is to be expected because many factors may influence the bacterial population level. The main thing to note from this data is that in all cases the decrease in the GNB count is apparent as storage time increased.

Table 18 shows the counts of GNB after 3 1/2 mo of storage of three cottons with known moisture contents (moisture contents of 5.18 to 6.07 percent). In spite of repeated determinations (n=12), the standard deviations are large, reflecting the heterogeneity of cotton. Regardless, the decrease in viable counts of GNB is apparent with time.

Bacterial Counts During Long-Term Storage of Cotton, Bracts, and Cotton Dust

Classers' specimens of cottons were stored at room temperature in the research laboratory of Janet J. Fischer at the University of North Carolina for 55 mo and showed a reduction of a log in GNB counts at 8 mo and a slow decrease in counts after that. Also the counts for gram-positive bacilli (GPB) decreased but at a slower rate. Hence cottons that originally contained more GNB than GPB had about equal counts after 2 yr, and the GPB eventually outnumbered the GNB (table 19).

In another study 12 bales of cotton were sampled in the winter following the harvest and at subsequent times of 5 mo and 29 mo. Some of the data from the study are shown in table 20, and again a trend is evident, showing a marked decrease in counts of GNB during storage. An analysis of variance (two-factor without replication) showed that the data for the individual samples (in a row) taken on the three dates are not significant (p=0.1372) but are very significant (p=0.0000014) when the data from the entire column are combined and compared. Comparison (by analysis of variance) of January 1985 to May 1985 has a p value of 0.00256. Comparison of May 1985 to May 1987 has a p value of 0.00000911. Again the individual samples (across by row) are not significant at the .05 level. After 29 mo of storage, only 1.7 to 12.1 percent of the GNB remained viable. However, the endotoxin-containing cell wall material of the GNB was still present because endotoxin levels did not change (data not shown). Extractable endotoxin (measured by the test for LPS) represents only a small amount of total endotoxin.

Levels of GNB also decline on bracts during storage (table 21). Usually the counts of viable GNB at 30

Table 20. Levels of GNB and lipopolysaccharides (LPS) in 12 bales of cotton sampled over 2 yr (means of three replicates for each point)

	January 1985		May 1985		May 1987*
Bale	GNB (millions cfu†/g)	LPS (μg/g)	GNB (millions cfu/g)	LPS (μg/g)	GNB (millions cfu/g)
1	4.3	70	2.6	70	0.33
2	7.1	100	2.5	100	0.86
3	2.7	400	2.3	100	0.14
4	4.3	400	0.76	100	0.12
5	1.2	100	0.37	100	0.10
6	1.3	400	1.2	400	0.04
7	9.1	400	2.9	100	0.50
8	5.5	100	3.9	100	0.18
9	11.0	100	2.4	100	0.19
10	5.4	100	2.7	40	0.11
11	7.1	400	2.4	100	0.09
12	1.6	100	1.1	100	0.09

*LPS for May 1987 was run by a different method and was therefore not comparable to previous LPS data and is not included in the table.

† *cfu*, Colony forming units.

Table 21. GNB on bracts collected throughout the harvest period and analyzed at 3 mo and 2 1/2 yr after harvest

	GNB (in millions of cfu*/g)	
Date of harvest	3 mo after harvest	30 mo after harvest
9/09	9.6	0.97
9/17	3.6	0.026
9/24	10.0	1.9
10/01	16.0	0.18
10/05	9.0	1.7
10/10	30	2.5
10/15	28	2.8
10/22	19	2.3
10/31	140	7.2

**cfu*, Colony forming units.

mo are about 10 percent of those at 3 mo after harvest. Two specimens (those harvested 9/17 and 10/01) had about 1 percent of the viable GNB remaining at 30 mo.

Karol et al. (1989) reported that cotton dust changes its content of microorganisms when stored for 4 yr. Originally multiple bags, each containing 3–5 kg of the same dust specimen, were stored at 10 °C in 1983. The dust was used repeatedly and in 1987 several bags were found to have lower-than-expected acute toxicity. The seven remaining bags were studied. Dust samples from two bags were less toxic and from one bag more toxic in the animal model. During storage there was an increase in the percentage of *Pseudomonas syringae* compared to *Enterobacter agglomerans*, an increase in the number of viable fungi, and a decrease in the number of viable corynebacteria. Samples from three bags of dust had markedly lower counts of GNB. Unfortunately, dust samples from the individual bags were not analyzed when the dust batch was divided for storage. However, all of the dust was from the same batch, and presumably all dust samples in individual bags had bacterial levels that were comparable to those for the one bag that was analyzed 4 yr earlier.

Sequential studies of dusts from Dr. Karol's laboratory (specific source bag not identified) revealed marked differences in counts of GNB and levels of LPS (lipopolysaccharide, the active component of endotoxin). One specimen of the original dust stored in a desiccator at room temperature in our laboratory showed a decrease in GNB, a decrease in percent of *E. agglomerans*, a decrease in corynebacteria, and an increase in fungal counts over a period of 1 yr. In conclusion cotton dust is heterogeneous, and levels of microbiological flora are not stable over time. If cotton dust is stored for experiments, baseline data must be reestablished at the time of each experiment. This is also true for cotton samples, as shown by the data presented in this paper.

Chun and Perkins (1991) studied baled cotton stored in a warehouse in Clemson, SC. They studied different bales over different time periods rather than studying one bale repeatedly over a period of years. The average viable GNB count decreased

Table 22. Viable bacteria in bales stored 0 to 11 yr

Storage period (yr)	No. of bales studied	Total bacteria	GNB	
			Log*	Percent
0	4	6.6	5.9a†	32.0
1	15	6.6	6.4a	67.1
2	4	5.7	3.1bcd	0.3
3	3	5.8	4.1b	11.7
4	3	5.3	3.6bc	2.6
5	13	4.5	1.9de	0.4
6	23	5.3	2.5cde	4.4
7	8	5.5	1.8de	0.1
8	4	6.5	3.1bcd	0.1
10	9	4.7	2.2cde	4.8
11	6	5.8	1.2e	0.9

*Log (cfu+1)/g was used to avoid taking a log of 0.
†Duncan's multiple range test (5 percent level).

Source: Chun and Perkins (1991).

with time and decreased much more so than counts of total bacteria (table 22). In storage years 2 through 11, the difference between total bacterial counts and gram-negative bacterial counts was significant (0.05 level). They concluded that studies of stored cottons may not detect the GNB if an extract of the specimen is studied. For example, a gram of cotton in 100 ml of diluent sampled with a loop that plates 0.1 ml may not permit detection of GNB if the GNB count is 3.0 logs or less. This may explain why some investigators who have found many bacteria have found no GNB. The GPB persist at detectable levels much longer and may be the predominant flora originally present in the cotton.

Bacterial Counts in Freshly Harvested Cotton From Different Areas

One final question remains. What are the relative proportions of GNB and GPB at harvest of cotton in the field? Fischer et al. (1989) used dried, sterile cotton to "pickup" organisms in the field; the cotton was tied onto the cotton plants as near as possible to the cotton bolls. They showed that the total bacterial counts on the experimental cottons grown in Lubbock, TX, and Raleigh, NC, were greater than the counts of GNB. Borbon-Reyes et al. (1988) showed the same thing for cottons grown in 1986 in Mississippi. Heintz et al. (1988, 1989) showed that total bacterial and GNB counts on cotton grown in Lubbock, TX, were almost the same (total counts were slightly higher). Batson et al. (1991) showed the same relationship between the counts of total bacteria and GNB on cottons grown in Mississippi in 1987 and 1988. Grice and Heintz (1990) also showed that the total bacterial counts exceeded the counts of GNB throughout the growing season on leaves of cotton plants.

Care must be taken in comparing counts of GNB and counts of GPB. If these counts are done separately and selective media are used to facilitate counting, the sum of the counts may exceed the total viable bacterial count done on the same specimen (same dilution). When total counts are made on one plate, there may be a large experimental error due to inhibition of one species of bacteria by another. This inhibition has been graphically illustrated when *Bacillus* species were grown in the presence of GNB (Fischer and Foarde 1991).

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Chapter 8. Effects of Cultural Practices on Bacteria in and on Cotton

A.A. Bell

The development of bacterial populations in and on cotton tissues has been the subject of research periodically for more than 65 yr. Several bacteria, *Xanthomonas campestris* pv. *malvacearum*, *Erwinia aroideae* (= *Erwinia caratovora*), *Erwinia herbicola* (= *Enterobacter agglomerans*), *Bacillus pumilus*, and *Bacillus subtilis* have been reported to cause boll rots and grow extensively on immature cotton fiber, reaching populations of 1 billion per gram of tissue or higher (Cauquil 1975, Watkins 1981).

Most bacteria in this group invade only bolls that have faults or wounds in the carpel wall. The wounds most often are caused by insects, such as boll weevils, plant bugs, or bollworms, but also may result from hail, harvest machinery, and freezing. The bacterium *X. c. malvacearum* also invades water-congested stomata of intact leaves, stems, and bolls and then extensively infects the subepidermal tissues, causing angular necrotic spots and blight on leaves and cankers on stems and bolls. The resulting disease is known as angular leaf spot or bacterial blight of cotton. It is a major cause of disease losses of cotton in most areas of the world where cotton is grown under moderate to heavy rainfall. Extensive studies on the epidemiology of *X. c. malvacearum* have been reviewed by Davis and Sandidge (1977).

Various saprophytic bacteria also grow on the surface of the cotton plant, and populations of these bacteria may increase with the senescence of various tissues. Population densities of 0.1 to 10.0 million per gram of dry fiber, leaf, or flower are common in most parts of the United States except in the San Joaquin Valley of California, where population densities generally are only one one-thousandth to one-tenth of those in other areas (Berni et al. 1988, Reyes et al. 1988, Fischer et al. 1989b, Heintz et al. 1989). Densities (per gram) on roots, stems, and branches are usually only one-fifth to one-tenth of those on fiber, whereas densities on bracts are 10 to 100 times greater than those on fiber (Fischer et al. 1988, Akinwunmi et al. 1989, Grice and Heintz 1990).

In most areas and years, more than 90 percent of the total bacterial population is made up of gram-negative species. The predominant gram-negative species in the United States are *E. agglomerans*, *Pseudomonas syringae*, and *Flavimonas oryzihabitans* (= *Pseudomonas* Ve2), and these species occur on all parts of the cotton plant. The predominant gram-positive bacteria are *Bacillus* and *Clavibacter* (also known as *Corynebacterium*) species (Rylander and Lundholm 1978; Millner et al. 1984; Simpson et al. 1988, 1989; Akinwunmi et al. 1989; Fischer and Foarde 1989; Grice and Heintz 1990). Other common gram-negative species on cotton lint are *Enterobacter cloacae*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas denitrificans*, *Pseudomonas maltophilia*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas aeruginosa*, and *Acinetobacter calcoaceticus*. More than 50 additional species of bacteria occur sporadically on cotton fiber.

Concentrations of gram-negative bacteria (GNB) in cotton dust have been correlated with acute respiratory responses of humans exposed to the dust (Rylander and Lundholm 1977, Cinkotai and Whitaker 1978, Fischer and Kylberg 1983, Millner et al. 1984, Fischer and Jacobs 1985). Even higher correlations occur between concentrations of endotoxin (from the GNB) in the dust and human responses (Olenchock et al. 1983, Castellan et al. 1984, Olenchock et al. 1984, Olenchock et al. 1985). These observations provide evidence that endotoxins from GNB on raw cotton fiber are at least one of the contributing factors to byssinosis.

Only limited information is available on the specific activity of endotoxins from different GNB in cotton dust. Strains of *E. agglomerans* (or *E. herbicola*) from grain dust and cotton produce potent endotoxins (Dutkiewicz 1976, Helander et al. 1980, Dutkiewicz and Kus 1983, Dutkiewicz et al. 1988). However, a tenfold difference in activity was found in preparations from different strains. Activities shown for *E. agglomerans* endotoxin include the following: lethal reaction in mice; lethal reaction in chick embryo; recruitment of macrophages, eosinophils, neutrophils, and lymphocytes in guinea pig lungs; and primary inflammatory lesions in rabbit skin. Lipopolysaccharides (LPS) from *P. putida* and *P. syringae* also are active as endotoxins in guinea

pig assays, whereas LPS from *Agrobacterium* and *Xanthomonas* are inactive (Helander et al. 1980, Ogundiran et al. 1986, Fischer and Foarde 1987). Toxins from gram-positive bacteria in cotton dust generally are much less active than those from GNB (Tuxford and Hoult 1988).

Ecology and Epidemiology of Bacteria

The development of bacterial populations on the cotton plant depends on the inoculum source and dissemination method of the bacteria, the availability of moisture and nutrients, the physical environment of the bacteria, and biological interactions between the bacteria and other microorganisms. These factors are discussed in the text that follows. Most studies of epidemiology have been concerned with the bacterial blight pathogen *X. c. malvacearum*. However, conditions that are most important for this bacterium are not necessarily the same as those that affect the saprophytic bacteria.

Inoculum Sources

Davis and Sandidge (1977) reviewed the epidemiology of *X. c. malvacearum* on cotton. Infested crop residues and seed serve as initial inoculum sources of *X. c. malvacearum*. This bacterium survives for many years when embedded in its own dry slime or in dry infected leaves, stems, bolls, or seed. When moist, however, the bacterium readily succumbs within weeks to environmental stresses, such as heat, desiccation, and antagonists. Consequently, infested dry cotton plant residues on the surface of fields or infested planting seed are essential for survival of the pathogen from one growing season to the next in temperate climates. Cultural practices used to control *X. c. malvacearum* are designed to facilitate the complete breakdown of crop residues before new seed are planted and to provide noninfested planting seed. These practices eliminate or greatly reduce the infection of cotton seedlings.

Saprophytic bacteria also survive on dry crop residues and fiber for many years (Karol et al. 1989, Chun and Perkins 1991). The predominant gram-negative bacterial species are found in soil (Berni et al. 1988) and on all plant parts immediately after emergence of seedlings (Grice and Heintz 1990), indicating that these bacteria survive freely in soil or on organic matter in soil in abundant numbers. Among the saprophytes only *E. agglomerans* is reported to occur within cotton seed (Akinwunmi et al. 1989). This may explain why the percentage of this bacterium in the total population is greatest early in the season and then often declines while populations of *P. syringae* and *F. oryzihabitans* increase late in the growing season. Cultural practices probably are not practical for reducing the initial inoculum of saprophytic bacteria because the bacteria survive readily in soils wherever cotton is grown.

Moisture Requirements

Moisture is the single most important variable affecting bacterial population densities on cotton foliage and fiber and in dust generated during processing of fiber. Moisture is critical for the multiplication, dissemination, and survival of both pathogenic and saprophytic bacteria. Cotton fiber (including the bracts and dust in the fiber) from the San Joaquin Valley of California generally has much lower bacterial densities than cotton in the rest of the United States (Fischer et al. 1988, 1989a, 1990; Fischer and Foarde 1991a). The San Joaquin Valley also has the lowest relative humidity and least rainfall during the period of boll opening before harvest, and bacterial blight does not exist in the valley, except for very isolated occurrences that originate from introduction of infested seed coupled with sprinkler irrigation (Schnathorst et al. 1960, Schnathorst 1968).

Several observations indicate that moisture as rain, dew, or humidity is the primary source of differences in densities of bacteria on fiber among geographical areas. When cotton was sheltered from rain and dew in a field at Lubbock, TX, bacterial densities on sheltered cottons compared with those on cotton exposed to rain and dew greatly decreased (Heintz et al. 1990). Likewise, in Mississippi, densities of bacteria sprayed onto fiber rapidly decreased on plants sheltered from rain and dew, whereas densities remained stable or increased on plants exposed to normal rain and dew (Batson et al. 1991). In the San Joaquin Valley, repeated sprinkler irrigation during boll opening caused bacterial densities to increase substantially on fiber (Harding, personal communication 1990). In each case,

bacterial densities were 10 to 1,000 times greater when plants were exposed to free moisture as dew, rain, or irrigation than when they were protected.

Efforts have been made to define critical moisture levels for bacterial survival and multiplication. Maximum disease development from *X. c. malvacearum* requires relative humidities above 85 percent at atmospheric temperatures of 30 to 36 °C (Davis and Sandidge 1977). DeLucca et al. (1990) found that population densities of *E. agglomerans* sprayed onto open cotton bolls declined rapidly at 11 percent relative humidity, remained stable at 32 and 44 percent relative humidity, and increased 10 to 100 times at 75 to 93 percent relative humidity. Zuberer and Kenerley (1991) found that increasing the fiber moisture content of air-dried fiber by as little as 2 percent could allow appreciable growth of bacteria, although increasing it by 10 percent usually was required for maximal bacterial densities. Fiber moisture levels greater than 10 percent develop during rains. Fiber also contains appreciably more than 10 percent moisture when bolls first open; therefore high humidity during boll opening can prevent drying and can allow extensive bacterial multiplication.

Nutrients

Pathogenic and saprophytic bacteria vary significantly in how they obtain nutrients. *Xanthomonas campestris* pv. *malvacearum*, like other plant pathogenic bacteria, produces pectolytic enzymes that apparently are important for obtaining nutrients from live host cells (Watkins 1981, Bell et al. 1986). *X. c. malvacearum* often reaches population densities greater than 1 billion per gram of diseased host tissue on a susceptible cotton cultivar but reaches densities of less than 10 million per gram on a resistant cultivar (Essenberg et al. 1990). The resistance response of cotton plants apparently is triggered by the pectinase enzymes of *X. c. malvacearum* and involves the production of enzyme-denaturing tannins and bactericidal phytoalexins, 2,7-dihydroxycadalene and 2-hydroxy-7-methoxycadalene. The resistance responses apparently are specifically targeted against the parasitic growth of the bacterium and may not affect saprophytic growth on leaves, stems, or fiber.

Saprophytic bacteria do not produce pectolytic enzymes and probably do not trigger cotton defense reactions. The major sources of nutrients for the saprophytes are amino acids and sugars exuded by cotton cells and various organic debris—such as pollen grains, flower petals, microbial cells, and "honey dew"—that accumulate on plant surfaces (Windels and Lindow 1985). Exudates from cotton cells may originate from the nectaries on cotton leaves and flower buds, from leakage of intact live cells of the epidermis, or from leakage of senescent parts such as flower petals and bracts. The cotton flower normally appears for 1 day only, and then the petals immediately undergo drying and senescence. The flower is usually shed after only a few days, but it can remain attached to the boll even when the boll is mature. The bracts that subtend the boll normally begin senescence prior to boll opening and support the greatest bacterial density of any cotton tissue (Akinwunmi et al. 1989, Fischer et al. 1989a). Thus, both the senescing flower and bract are potentially important sources of nutrients for bacteria on bolls and fiber. Organic debris apparently is also an important source of nutrition because artificial bolls made from washed cotton and hung from plants in the field support bacterial densities nearly as great as those on natural bolls (Fischer et al. 1989a, 1990).

The cotton fiber presents an unusual situation as far as nutrients are concerned. A fiber is a single cell that protrudes from the epidermis of the seed coat, and fiber has a prolonged period of growth during which cellulose fibrils are deposited daily into the secondary wall for several weeks. The mature fiber cell is 90 percent cellulose and consequently has a very high ratio of carbon to other elements. For example, the concentrations of nitrogen, phosphorus, calcium, magnesium, sulphur, iron, and boron in mature fibers are equal to or less than one-tenth of those in bracts, leaves, or other parts of the plant (Marcus-Wyner and Rains 1982). Also, as the boll matures elemental nutrients are transferred from the fiber cells to the seed embryo so that the nutrient content of a single immature fiber cell declines as more cellulose is synthesized (Leffler 1986). Maximum concentrations of elemental nutrients in fibers occur in bolls that are 20 to 35 days old. Domelsmith (1988, 1989) showed that there is a large decrease in free sugar concentrations in fibers

during the last few days before the boll opens. These facts probably explain why bacteria multiply to much higher densities on immature fibers than on mature fibers and why densities are greater on mature bracts than on mature fibers. The low concentration of nitrogen in mature fibers is probably especially important in limiting bacterial multiplication.

Dissemination

Bacteria in small particles of dry slime or infected plant debris are readily disseminated by wind. *Xanthomonas campestris* pv. *malvacearum* and *E. agglomerans* also are disseminated in infested planting seed. Rain, dew, irrigation water, insects, and machinery also provide important means of dissemination for all bacteria. Cultural practices such as using furrow irrigation in place of sprinkler irrigation, using skip-row planting, and keeping machinery out of the field while dew is present can limit dissemination of *X. c. malvacearum*. However, these practices are of little value in preventing dissemination of the major saprophytic bacteria, which are universally present in soils and extensively inhabit the surfaces of most cotton seedlings by the time they emerge from the soil (Berni et al. 1988, Grice and Heintz 1990).

Physical Requirements

Temperature and sunlight are the most important components of the physical environment that affect bacteria. Hydrated bacteria are readily killed when directly exposed to sunlight, unless they are suspended in water. In dry slime or plant tissue, bacteria are much more resistant to sunlight. The bacterial blight pathogen is favored by air temperatures of 30 to 36 °C and by soil temperatures of 21 to 26 °C (Davis and Sandidge 1977). *Enterobacter agglomerans* is better adapted to high temperatures and dry conditions than either *P. syringae* or *F. oryzihabitans* (Fischer and Foarde 1991b). As a result, the ratio of *E. agglomerans* to other GNB generally is greatest during summer and in geographical areas characterized by relatively high temperatures. *Flavimonas oryzihabitans* populations as a percentage of the total population are variable or sporadic during the growing season; this saprophyte is displaced by *P. syringae* at 25 °C but displaces *P. syringae* at 40 °C (Fischer and Foarde 1991b). Temperature does not appear to have any appreciable effect on the total densities of combined gram-negative species.

Biological Interactions

The relationships between bacteria and other microorganisms on cotton foliage and fibers is not well defined. Bacteria antagonistic to *X. c. malvacearum* have been isolated from cotton, and one of these was identified as a *Flavobacterium* species (Davis and Sandidge 1977). However, no antagonists of the major saprophytic bacteria have been found on cotton. On other crops, strains of *P. fluorescens* and *E. herbicola* have antagonized ice-nucleating strains of *P. syringae*. Likewise, ice-nucleation inactive strains of *E. herbicola* or *P. syringae* have been used largely to displace ice-nucleation active strains of the same species (Windels and Lindow 1985). *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus thuringiensis*, all of which occur on cotton fiber, also produce antibiotics that are active against various microorganisms (Baker et al. 1983). The effectiveness of antibiotics from *Bacillus* against the saprophytic bacteria on cotton, however, is unknown.

Cultural Practices for Controlling Bacterial Diseases

Bacterial diseases of plants generally are controlled by a combination of cultural practices. These practices are designed to take advantage of weak spots in the ecology and epidemiology of the target bacterium. Practices used to reduce bacterial diseases include the use of resistant cultivars, water and fertilizer management, adjustments in tillage and harvest practices, and application of biocontrol agents. From 1985 to 1989 my colleagues and I examined how some of these cultural practices affect populations of saprophytic bacteria on raw cotton fibers (Bell et al. 1987, 1990; Bell and Tribble 1988, 1989). These and other related studies are discussed in the sections that follow.

Resistant Cultivars

The most widely used practice to control bacterial blight of cotton is the growth of resistant cultivars.

Sixteen blight-resistance genes that reduce bacterial blight severity have been identified, and several of these have been transferred into modern cultivars to provide protection against bacterial blight (Bird et al. 1979, Watkins 1981). These genes generally reduce populations of *X. c. malvacearum* in inoculated leaves and cotyledons to densities only 10.0 to 0.1 percent of those in susceptible cultivars (Essenberg et al. 1990). The exact amount of reduction depends on the specific gene or gene combination for resistance and the genetic background in which the genes are present. The resistance restricts the parasitic growth of *X. c. malvacearum* on internal cells of leaves, stems, and bolls; it is not known how it affects epiphytic growth of bacteria on the surfaces of cotton plants.

Bell and colleagues (Bell et al. 1987; Bell and Tribble 1988, 1989) measured bacterial populations of raw cotton from 22 cultivars and lines grown for 3 yr successively in the Brazos River Valley near College Station, TX. The cultivars and lines varied in levels of resistance to bacterial blight and in occurrence of mutant morphological characters (glandless, nectariless, frego bract, okra-leaf, smooth stem, and stormproof boll) known to affect the incidence of insect damage to bolls and the incidence of boll rots caused by fungi. In 1987, 14 additional cotton lines with white, tan, brown, or green fiber were compared to determine any effects of fiber color on bacterial populations. The results of these studies are given in tables 23 to 27.

The bacterial blight resistance genes did not reduce population densities of total or GNB on cotton fiber. Over the 3-yr period 8 blight-resistant cultivars had a mean total bacterial population of 1.87 million per gram of fiber compared to 1.52 million per gram for 13 susceptible cultivars (tables 23 to 25 and 27). The 23 percent greater density of bacteria on the blight-resistant cultivars was consistent for each of the 3 yr and was statistically significant in 1987. There was no significant difference in the percentage of GNB in the total population (86 percent for the blight-resistant cultivars and 90 percent for the susceptible cultivars).

The only morphological characters that were associated with reduced densities of bacteria were the frego bract and okra-shape leaf characters (table 23). The density of total bacteria on isolines with either of these characters was only 62 percent of that on the isoline with normal bracts and normal leaves over the 3 yr (table 23). The percentage of bacteria that were gram-negative also was reduced from 89 percent on the normal isoline to 87 percent and 84 percent on the isolines with okra leaf and frego bract, respectively. None of the differences were statistically significant (p=0.05), but they are consistent with the known reduction of boll rots associated with these two morphological characters (Roncadori et al. 1975). Also, Millner and Jones (1987) found that after an extended period of rainy weather, endotoxin concentrations on fiber were reduced significantly by the okra leaf and frego bract characters even though viable counts were not reduced significantly.

Both the smooth and nectariless characters reduce damage from bollworms and budworms (Bell 1984). Therefore, cultivars with these characters might have decreased numbers of bacteria because fiber from insect-damaged locules has higher densities of GNB and concentrations of endotoxin than fiber from undamaged bolls (Simpson et al. 1983, Millner et al. 1987). Contrary to expectations, though, the smooth and nectariless characters were associated with increased bacterial densities (table 23). The B-smooth isoline had a mean of 2.65 million total bacteria per gram of fiber compared to 1.36 million per gram for the B-hairy isoline, which has a normal distribution of stem and leaf hairs. The increase in bacterial density associated with the smooth character was statistically significant for 1985, 1987, and the 3-yr means.

The isolines for leaf hairs had similar percentages of GNB: 90 percent for B-smooth and 88 percent for B-hairy. The nectariless cultivars Stoneville 825 and Tamcot GN–8 had a mean of 1.65 million bacteria per gram of fiber compared to 1.07 million per gram for the similar nectaried cultivars Stoneville 213 and Rogers GL–6. Only the difference between Tamcot GN–8 and Rogers GL–6 in 1985 was statistically significant. The nectaried and nectariless cultivars had 90 and 91 percent GNB, respectively, in the total populations. Columbus and Fischer (1986) also found 20 to 35 percent increases in gram-negative bacterial densities associated with the smooth and nectariless characters in Mississippi, but the differences were not statistically significant. Therefore,

the smooth and nectariless characters do not appear to have any promise for reducing GNB on fiber.

Although the smooth and nectariless characters confer resistance to bollworms and budworms, these characters are not effective against bollweevils, sucking insects, or plant bugs. It may be that the small wounds produced by the latter insects are more effective in selectively introducing bacteria into the locules of immature bolls than are the more massive wounds caused by bollworms and budworms. In this respect, it is interesting to note that bacterial boll rots in Africa are often associated with punctures from the plant bug *Dysdercus volkeri* (Cauquil 1975), and *Erwinia*-induced boll rot in California is associated with damage caused by the stinkbug, *Euschistus impictiventris* (Watkins 1981). Millner et al. (1987) found that the greatest endotoxin concentrations and gram-negative bacterial densities in locks of cotton from South Carolina were associated with fluffed locks that had a yellow-spotted base.

The base of the young boll also is a common puncture site for the boll weevil, which can introduce the bacteria. Some insect wounds may favor introduction of bacteria into immature bolls, whereas wounds from other insects may favor fungal boll-rotting pathogens. The relative efficiencies of different insects for inoculating immature bolls with GNB needs to be ascertained before we can accurately predict the value of different insect control procedures for reducing endotoxin contamination of fiber.

As expected the stormproof boll, compared to the normal boll, was consistently associated with increased densities of total and GNB on fiber (table 24). The cultivar GSA–71, which has a stormproof boll, also consistently had greater bacterial densities than the normal cultivars Lankart 57, Acala C1, Acala SJ–5, and Deltapine 61 in each of the 3 yr, although only the difference between GSA–71 and Lankart 57 or Deltapine 61 in 1985 was significant (p=0.05) (table 27). Greater concentrations of GNB and endotoxin on fiber of GSA–71, compared to Deltapine 61 and Acala SJ–5, also were found with cotton produced in Mississippi in 1982 and 1984 (Fischer and Sasser 1987). The glandless line

Table 23. Concentrations of GNB on raw cotton fiber from cotton lines varying in resistance to insects

Cotton cultivar or line*	Mutant resistance character†	Bacterial blight reaction‡	Colony-forming units per gram (millions)			Percentage GNB in total bacteria		
			1985	1986	1987	1985	1986	1987
TAM–T1–3	none	1.0	4.97	0.93	0.90	93	89	84
TAM–T1–1	ok	1.0	2.48	0.78	0.78	91	85	77
TAM–T1–2	ok,fg	1.0	3.10	0.89	1.03	83	87	82
TAM–T1–4	fg	1.3	2.15	1.05	0.84	88	87	85
TAM–B–Nm	none	1.5	1.93	0.65	1.04	89	85	91
TAM–B–Sm	sm	1.3	5.21§	0.94	1.29§	97	85	88
STO–213	none	4.0	2.78	0.48	0.91	94	88	91
STO–825	ne	4.8	3.45	0.47	0.80	95	89	86
R–GL6	none	4.5	0.76	0.75	1.02	87	91	90
TAM–GN8	ne	2.0	2.62§	0.86	1.05	98	88	91

**TAM*, Tamcot. *STO*, Stoneville. *R*, Roger. Cultivars or lines with mutant characters are compared with the first one above them that has no mutant character.
†Morphological characters: *ok*, okra leaf; *fg*, frego bract; *sm*, smooth stem; *ne*, nectariless.
‡Reaction scale: 1 = immune to 5 = susceptible.
§Difference from comparable normal line is significant (p=0.05).

Table 24. Concentrations of GNB on raw cotton fiber from cotton lines varying in boll character

Cotton cultivar or line*	Boll character	Bacterial blight reaction†	Colony-forming units per gram (millions)			Percentage GNB in total bacteria		
			1985	1986	1987	1985	1986	1987
LKT–57	Normal	4.8	1.09	0.61	1.03	95	86	90
PAY–202	Stormproof	3.3	1.71	0.84	1.00	98	88	97
GSA–71	Stormproof	4.0	3.57‡	0.77	0.86	95	87	92
R–GL6	Normal	4.5	0.76	0.75	1.02	87	91	90
PAY–784	Stormproof	4.0	3.81‡	0.87	1.23	96	87	93

*LKT, Lankart. PAY, Paymaster. R, Roger. Cultivars with stormproof bolls are compared with the first cultivar above them that has normal bolls.
†Reaction scale: 1 = immune to 5 = susceptible.
‡Difference from comparable normal line is significant (p=0.05).

Paymaster 784, which has a stormproof boll, also consistently had higher bacterial densities on raw cotton fiber in each of 3 yr than the other glandless cultivars or lines, Acala 8160, Tamcot GN–8, Roger GL–6, and Stoneville 209 (tables 24 and 25). The differences between Paymaster 784 and Roger GL–6 or Stoneville 209 in 1985 and Paymaster 784 and Stoneville 209 in 1987 were significant. The cultivar Paymaster 202, which also has a stormproof boll, had greater bacterial densities in 10 of 12 comparisons with the cultivars Acala SJ–1, Tamcot CAMD–E, Lankart 57, and Stoneville 213, which had normal bolls (table 25). The mean increase in total bacterial populations on fiber due to the

Table 25. Concentrations of GNB on raw cotton fiber from cotton lines having glanded or glandless foliage

Cotton cultivar or line*	Foliage type	Bacterial blight reaction†	Colony-forming units per gram (millions)			Percentage GNB in total bacteria		
			1985	1986	1987	1985	1986	1987
PAY–202	Glanded	3.3	1.71	0.84	1.00	98	88	97
PAY–784	Glandless	4.0	3.81	0.87	1.23	96	87	93
Acala-SJ1	Glanded	4.5	2.51	0.75	0.85	91	92	90
Acala-8160	Glandless	5.0	1.69	0.67	1.00	94	86	94
TAM–CAMD–E	Glanded	2.0	1.17	0.70	1.15	93	86	89
TAM–GN8	Glandless	2.0	2.62	0.86	1.05	98	88	91
LKT–57	Glanded	4.8	1.09	0.61	1.03	95	86	90
R–GL6	Glandless	4.5	0.76	0.75	1.02	87	91	90
STO–213	Glanded	4.0	1.13	0.48	0.91	93	88	91
STO–209	Glandless	5.0	0.78	0.62	0.72	96	88	87

*PAY, Paymaster. TAM, Tamcot. LKT, Lankart. R, Roger. STO, Stoneville. Cultivars within each of the five pairs are genetically similar and comparable. None of the differences between lines or cultivars within palrs were significant (p=0.05).
†Reaction scale: 1 = Immune to 5 = susceptible.

Table 26. Concentrations of bacteria and endotoxins on white, tan, brown, and green raw cotton fiber*

Genetic background of cotton lines	Lint color	Total bacteria (millions/g)	Percentage GNB in total bacteria	Endotoxin (ng/g)
Tamcot CAMD–E	White	1.85b	90b	315b
	Tan	1.89b	90b	308b
Deltapine 14 (TM–1)	White	1.47a	87c	232a
	Brown	3.28c	91b	245a
	Green	3.46d	94a	304b

*Numbers followed by the same letter within a column are not significantly different (p=0.05).

stormproof boll character was 53 percent, and cultivars with stormproof bolls had 93 percent GNB compared to 90 percent on cultivars with normal bolls. In 1987 the percentages of GNB on the lines and cultivars with stormproof bolls (92–97 percent) were significantly greater than the percentages of GNB on many cultivars with normal bolls (77–90 percent). The semiclosed boll of cultivated Asiatic cotton, *Gossypium herbaceum*, compared to the normal boll of the American Upland cotton, *Gossypium hirsutum*, also increases the numbers of bacteria and concentrations of endotoxins in fibers (Morey et al. 1985). The stormproof and semiclosed bolls hold locks in the boll more tightly together and probably increase moisture retention in the fiber. This apparently allows more bacterial growth, especially of the GNB.

The glandless character also was expected to increase densities of bacteria on fiber because this character results in more damage from bollworms, budworms, leafworms, beetles, and lygus bugs (Bell and Stipanovic 1977). However, comparisons of each of five glandless cultivars with similar glanded cultivars for 3 yr showed only a 14 percent increase in total bacteria and no change in the percentage of GNB associated with the glandless character (table 25). In no case was the difference between the glandless and the comparable glanded cultivar or line significant (p=0.05). Fibers from the glanded and glandless cultivars or lines mentioned in table 25 were sent to Fischer and Foarde, and they did not find any appreciable difference in concentrations of GNB or endotoxin associated with the glandless character (Fischer and Foarde 1989). The failure of the glandless character to greatly affect bacterial populations may indicate that bollworms and leafworms are less important than other insects for introducing GNB into immature bolls. The glandless character does not influence damage caused by sucking insects and has actually resulted in decreased damage from boll weevil (Bell and Stipanovic 1977).

The relationship of fiber color with bacterial populations and endotoxin content is shown in table 26. The tan and brown colors are due to different amounts of melanins formed from the oxidation of catechins and condensed tannins (Bell, unpublished results). The green fibers result from the daily deposition of suberin and wax (as many as 26 layers) between the cellulose layers in the secondary walls of the cotton fiber (Ryser and Holloway 1985). Both tannins and suberins denature enzymes and have been implicated in resistance to cotton diseases (Bell et al. 1986, 1992). However, in this study both the brown and green fiber characters were associated with greater concentrations of GNB and endotoxins on the fiber, and the tan fiber had no effect on bacterial populations or endotoxin (table 26). This indicates that the saprophytic bacteria do not depend on extracellular hydrolytic enzymes to obtain their nutrition. The enzyme-denaturing activities of the tannins and suberins, however, may interfere with the final polymerization and transport of simple sugars and amino acids in the fiber so that the fiber contains a higher nutrient base.

Table 27. Concentrations of GNB on raw cotton fiber from normal cotton cultivars*

Cotton cultivar or line	Bacterial blight reaction	Colony-forming units per gram (millions)			Percentage GNB in total bacteria		
		1985	1986	1987	1985	1986	1987
Deltapine 61	4.0a	1.62a	0.60a	0.70ab	96a	93a	88a
Acala SJ–5	4.5a	1.83abc	0.79a	0.78abc	92a	91a	88a
Acala C–1	4.5a	2.61bcd	--	0.60a	94a	--	79b
Acala SJ–1	4.5a	2.51bcd	0.75a	0.85abcd	91a	92a	90a
Stoneville 213	4.0a	2.78abcd	0.48a	0.91bcdef	94a	88a	91a
Lankart 57	4.8a	--	0.61a	1.03cdefg	--	86a	90a
Tamcot CAMD–E	2.0b		0.70a	1.15fgh	--	86a	89a
Tamcot B-Normal	1.5b	1.93ab	0.65a	1.04defg	89a	85a	91a
Tamcot T1–3	1.0c	4.97cd	0.93a	0.90cdefg	93a	89a	84a

*Numbers followed by the same letter within a column are not significantly different (p=0.05).

Although morphological characters account for some of the differences in numbers of bacteria per gram of fiber among cultivars, even greater significant differences occur among cultivars with normal morphological characters (table 27). The cultivar Deltapine 61 was especially noteworthy in our studies. Numbers of bacteria in raw cotton fiber of this cultivar were the lowest or next to the lowest of the nine normal cultivars or lines in each of the 3 yr; many of the differences between Deltapine 61 and other normal cultivars were significant (p=0.05). Deltapine 61, compared to Acala SJ–5 and GSA–71 cultivars, also had lower numbers of bacteria and concentrations of endotoxins when grown in Mississippi and the High Plains of Texas in 1982, 1983, and 1984 (Millner et al. 1984; Olenchock et al. 1983, 1984, 1985; Fischer and Foarde 1989). Likewise, cotton identified as Mississippi, Grade 41 [in fact Deltapine 61 (Morris and Catalano 1984)] had the lowest bacterial densities (Millner et al. 1983) and endotoxin concentrations (Olenchock et al. 1983) of several cultivars harvested from Mississippi and Texas in 1981 and before.

Dusts from Deltapine 61 were less active against humans than those from two other cultivars when the cottons originated from Mississippi and Texas (Olenchock et al. 1983, 1984, 1985). The cultivar Deltapine 50, which has largely replaced Deltapine 61 in cotton production, had the lowest bacterial densities of any of five cultivars grown in Mississippi in 1986 (Simpson et al. 1987c).

The Tamcot cultivars and lines (Tamcot CAMD–E, Tamcot GN–8, Tamcot B–Sm, Tamcot B–Nm, and Tamcot T1–1 to –4) developed from the MAR germplasm pool at Texas A&M University had the highest concentrations of bacteria on fiber of any of the cultivars with normal boll shape in our studies. Likewise Simpson et al. (1987c) found that Tamcot SP 37H had about 10 times greater bacterial counts on fiber than did Deltapine 50, Deltapine 61, and Acala 1517–75 harvested early (September 24) in Mississippi. These observations are in agreement with the suggestion that the multiple pest resistance of the MAR cultivars is at least partially due to their ability to support greater populations of beneficial bacteria on plant surfaces, especially roots (Bird et al. 1979). Sprays made up of live cells of *B. cereus* and *Bacillus megaterium* isolated from the leaves of MAR cultivars also have been used to suppress insect pests on these cultivars (Benedict and Bird 1981).

The reasons for different bacterial population densities on normal cultivars is not clear. However, Deltapine 61 has been shown to produce signifi-

cantly higher concentrations of lacinilene and cadalene phytoalexins in bract tissue than Acala SJ–5 and GSA–71 (Greenblatt and Bell 1986). The cadalenes and lacinilenes from cotton have bactericidal activity at less than 50 μg/ml of media (Essenberg et al. 1990), and 2,7-dihydroxycadalene is the most potent bactericide found in concentrated extracts from raw cotton fiber of Deltapine 61 grown in Mississippi (Bell, unpublished results). Concentrations of lacinilenes and cadalenes in bracts of Deltapine 61 often exceed 200 μg/g in bracts from Mississippi and Texas (Greenblatt and Bell 1986), and mean concentrations previously found in Deltapine 61, Acala SJ–5, and GSA–71 are negatively correlated with the bacterial densities found in these studies (tables 24 and 27). The Stoneville glanded-glandless pair, which had the lowest bacterial densities on fiber of any of the five pairs (table 25), also had the highest concentrations of lacinilene and cadalene phytoalexins in bracts in a previous study (Greenblatt and Bell 1986). These combined observations suggest that these natural bactericides may at least partially restrict growth and development of saprophytic bacteria in bracts and leaves, which make up a major part of the dust generated from cotton fiber.

Overall, studies of cultivars and breeding lines indicate that there is little or no potential for reducing bacterial contamination on fiber by plant breeding. The extremes of variation among all cultivars (0.81 to 3.97, 1.69 to 5.37, 0.53 to 1.21, and 0.76 to 1.47 million total bacteria per gram of fiber in our four different experiments over 3 yr) show little potential for genetic progress. Also, none of the breeding lines in any study had less bacterial contamination than the Acala, Deltapine, and Stoneville cultivars, which were developed previously for their high quality fiber and yield potentials and are being grown extensively in the United States.

Water Management

Although moisture is probably the most important factor affecting multiplication and survival of the GNB, few studies have evaluated the effects of water management practices on bacterial densities on fiber. The incidence of bacterial blight has been shown to be greatly increased by soaking seed in water, sprinkler irrigation, and improper handling of runoff water from irrigation (Davis and Sandidge 1977). Schnathorst et al. (1960) and Schnathorst (1968) found that 66 incidences of bacterial blight in the San Joaquin Valley of California from 1951 to 1959 were almost totally associated with the use of sprinkler irrigation in the western part of the valley. The disease normally was absent when furrow irrigation was used.

Because irrigation is usually discontinued before bolls begin to open, it is uncertain what effects sprinkler irrigation has on the bacterial populations of fiber. However, sprinkler irrigation definitely should affect the levels in leaf and bract trash contained within the raw fiber. In this respect it is interesting to note that mean bacterial densities on raw cotton from the Imperial Valley of California are about 10 times higher than those from the San Joaquin Valley of California (Simpson and Marsh 1985). Sprinkler irrigation is used much more extensively in the Imperial Valley than in the San Joaquin Valley. Thus, using furrow irrigation in place of sprinkler irrigation is probably an effective method for reducing bacterial contamination of fiber in arid climates where most of the water is supplied by irrigation.

Another practice that can appreciably affect microbial densities in raw cotton fiber is prolonged storage of seed cotton in trailers or in the field before ginning. Moisture concentrations in stored seed cotton are critical for microbial activity. When the moisture level of seed cotton is 10 percent or less, temperatures in stored cotton generally decrease slowly as air temperatures decrease, indicating there is little microbial activity. As moisture levels increase progressively above 12 percent in stored seed cotton, however, there is progressively more heating, indicating there also is progressively more microbial growth (Wilkes 1978, Curley et al. 1990). The heating is generally accompanied by increases in free fatty acids, decreases in oil content, and loss of viability in seeds (Cherry et al. 1979) and by increases of spotting and yellowing of fiber (Wilkes 1978, Curley et al. 1990).

The critical factors that affect yellowing of the fiber during storage are moisture content, days stored, average air temperature during storage, and the initial temperature of the cotton when stored (Curley et al. 1990). Plant trash (leaves, bracts,

stems, boll parts, and weeds) is an important source of moisture and can cause localized "hot spots" in stored seed cotton (Wilkes 1978). The microorganisms responsible for the heating of stored seed cotton have not been studied in detail. Cherry et al. (1979) found that population densities of *Aspergillus* species, *Alternaria* species, and *Mucor* species in seed were considerably higher after storage of seed cotton at 13 percent moisture compared to at 7 percent moisture; and bacterial numbers in seed increased greatly at either moisture level during storage in modules. Because gram-negative bacterial numbers are closely associated with yellow spotting in the field (Millner et al. 1987), it is probable that the marked increase of yellowing and spotting of fiber during storage of moist seed cotton is due, at least partially, to bacterial multiplication. The moisture concentration of seed cotton should be monitored closely during harvest to ensure storage at moisture levels below 12 to 13 percent. The stored seed cotton also should be protected from rain, which can further stimulate microbial growth in modules (Wilkes 1978).

The drying of fiber during ginning may be critical for preventing microbial growth in bales. Bargeron et al. (1986) found that bales formed with fiber containing 8 or 9 percent moisture had 50 to 75 percent higher concentrations of viable GNB after 3 mo than those formed with fiber having 7 percent moisture. They did not determine whether endotoxin concentrations were also affected. Consequently, it is uncertain whether survival or multiplication of the GNB was affected.

Nutrient Management

The severity of damage to the cotton plant by pests is dependent upon the nutritional status of the plant. High rates of nitrogen fertilization of cotton often stimulate growth and multiplication of pathogenic microorganisms (Bell 1989) and insects (Teague et al. 1986). Consequently, symptoms and damage from pests can be aggravated by nitrogen fertilization. Potassium deficiency, which causes accumulation of free amino acids in cotton tissues (Joham and Blevins 1969), also increases the severity of most cotton diseases (Bell 1989). Potassium fertilizers decrease disease severity when used to correct a deficiency but have no effect on disease severity when added to potassium-sufficient soils.

While studying the effects of plant genotype on saprophytic bacterial populations of fiber, Bell et al. (1987) and Bell and Tribble (1988, 1989) found significant differences in bacterial numbers per gram of raw fiber among cottons harvested from different blocks within the same field even though the blocks received the same treatments. Differences in soil moisture or humidity could not account for the differences in bacterial numbers. Analyses of nutrient elements in soil samples removed in a grid pattern from the field showed that only nitrogen concentrations had any correlation with bacterial densities on fiber.

In a subsequent study, Bell et al. (1990) examined relationships between rates of nitrogen fertilization and concentrations of GNB on raw cotton fiber. The results of this study for 1988 are shown in tables 28 and 29. Concentrations of total and GNB at the rate of 180 lb nitrogen per acre were significantly greater than those at the 60 and 120 lb rates (table 28). Increases in concentrations of total and GNB and percentages of GNB on fiber correlated significantly with increasing rates of nitrogen fertilization (table 29). Mean concentrations of nitrogen in fibers from plants receiving 60, 120, and 180 lb nitrogen per acre were 0.176 percent, 0.188 percent, and 0.193 percent, respectively. The percentages of nitrogen in fiber correlated more closely with concentrations of total or GNB than with rates of nitrogen fertilization. Thus, the significant differences among blocks that Bell and Tribble (1988,

Table 28. Concentrations of bacteria and yield of raw cotton fiber produced with variable nitrogen fertilization*

Fertilization rate (lb nitrogen/acre)	Millions of bacteria/g		Percent GNB in total bacteria	Yield of fiber (lb/acre)
	Total	GNB		
60	10.0a	5.1a	53a	872a
120	11.1a	6.6a	61a	940a
180	15.3b	10.5b	68a	877a

*Numbers followed by the same letter within a column are not significantly different (p=0.05).

Source: Bell et al. (1990).

Table 29. Correlation coefficients among nitrogen fertilization rates, nitrogen content of fiber, and bacterial numbers

	Percent nitrogen in fiber	Concentration of total bacteria	Concentration of GNB	Percent GNB
Rate of nitrogen per acre	+0.23*	+0.67*	+0.85*	+0.60*
Percent nitrogen in fiber		+0.61*	+0.40*	-0.05
Concentration of total bacteria			+0.87*	+0.17
Concentration of GNB				+0.61*

*Correlation is significant (p=0.05).

Source: Bell et al. (1990).

1989) observed in a single field could be due to differences in amounts of nitrogen accumulated in fiber. Differences in the nitrogen content of other plant parts, which become entrapped in the harvested seed cotton, also may influence population densities. Thompson et al. (1976) used nitrogen fertilization rate plus time in the growing season to account for 79 percent of the variation in nitrogen concentrations in leaves, buds, and bolls.

The effects of nitrogen fertilization rates on bacterial populations indicate that nitrogen as well as moisture is a limiting factor for bacterial concentrations in raw cotton fiber. Coefficients of determination calculated from the correlation coefficients in table 29 for the effects of nitrogen fertilization on total and gram-negative bacterial concentrations were 0.45 and 0.72, respectively; in other words, appreciable amounts of the variability in bacterial concentrations could be due to differences in the rates of nitrogen fertilization. The tenfold or greater densities of bacteria in bracts compared to fibers correspond to similar differences in nitrogen content. Raw cotton fiber contains less than 0.2 percent nitrogen (Bell et al. 1990), whereas mature bracts and leaves generally contain about 2.0 percent nitrogen (Thompson et al. 1976, Morey 1977, Raymer et al. 1979). The fact that cultivars differ significantly in their content of nitrates in petioles (Jenkins et al. 1982) further suggests that significant differences in nitrogen content in fiber may occur also among cultivars. Such differences could contribute to the differences in concentrations of bacteria among tissues. Excess nitrogen fertilization or other cultural practices that raise nitrogen concentrations in fiber or accompanying trash should be avoided.

Very little is known about how nutritional, environmental, and genetic factors other than nitrogen fertilization affect the nitrogen content of fiber. The ratio of ammonia and amino acids to nitrates and proteins also is probably important because some GNB do not have the enzymes necessary to use nitrate and protein as nutrients. At least some tissues of the cotton plant accumulate amino acids and ammonia in response to low temperatures (Hunter and Guinn 1968), potassium deficiency (Joham and Blevins 1969), and infection with the Verticillium wilt pathogen (Bell 1991). Thus, fertilization with potassium and use of early-maturing, disease-resistant cultivars might decrease bacterial numbers where potassium deficiency or Verticillium wilt problems occur. Other factors that might affect the concentration and availability of nitrogen in fiber include soilborne fungal pathogens, nematodes, sucking insects and mites, and harvest-aid chemicals.

Tillage, Pest Control, and Harvest Practices

There is no specific information on how planting and tillage practices affect bacterial contamination of fiber. However, any practice that facilitates boll

set and development in the United States probably is desirable. Bell et al. (1990) found that bolls set early in the flowering period in central Texas had significantly lower percentages and concentrations of GNB than bolls set 2 wk later after the peak of boll set. The bolls set early on June 28 opened in mid-August before temperatures had begun to decline appreciably and before humidity and rainfall had increased in late summer and fall, as they often do during the hurricane season. The decline in temperature and increase in moisture (rainfall incidence, dew period, and relative humidity) probably account for the qualitative difference in bacterial populations found on bolls set early and midway through the fruiting season. Gram-positive bacteria often are much more prolific than GNB in the warm, dry climate of the San Joaquin Valley of California both on fiber (Chun 1990) and bract (Fischer and Foarde 1991a).

Planting early, planting on raised beds, and planting in narrow rows are all practices that increase the percentages of cotton bolls that set early in the growing season and open during warm, dry weather. Planting in narrow rows may have the added advantage of decreasing nitrogen concentrations in tissues, since it has been shown to decrease nitrate concentrations in cotton foliage (Sunderman et al. 1979). All of these practices are likely to decrease the number of GNB on fiber, but they may not change total numbers of bacteria.

Certain insects, such as the boll weevil and plant bugs, puncture holes through the boll wall, allowing various fungi and bacteria to grow extensively on the immature fibers. Locks from insect-injured parts of the boll often have 100 to 10,000 times more GNB per gram of fiber than do locks from uninjured bolls (Simpson and Marsh 1982; Simpson et al. 1983, 1987a, 1987b; Millner et al. 1987). Other insects feed by sucking on plant tissues, and waste products from these insects ("honeydew") are a rich source of simple nutrients, especially sugars (Balasubramanya et al. 1985). Honeydew often accumulates on fiber of open bolls and is a major cause of "sticky" cotton. It also provides nutrients for microbial growth.

The exact effects of insect injury on bacterial concentrations in cotton mill dust remain uncertain because direct comparisons of bacteria on cotton (produced with different insecticide regimes or receiving different degrees of measured insect damage) are not available. Several observations indicate that the effects of insect injury may not be as great as would be expected in terms of the concentrations of bacteria in insect-injured locks. Millner et al. (1987) found that adding as much as 10 percent "tightlocks" to cotton caused only slight increases in gram-negative bacterial numbers and endotoxin content of cardroom dust. Similarly, Bell et al. (1987 to 89) found that glandless cotton lines, which suffer considerably more damage to bolls from bollworms and budworms than glanded lines, had only 14 percent greater concentrations of bacteria on fiber (table 25). Simpson et al. (1987b) found about 10 times greater concentrations of GNB on fiber from cotton grown on the upland farm compared to bacteria levels on fiber grown at a Brazos River bottom farm at College Station, TX. While part of this difference probably is due to the much greater boll weevil damage on the upland farm, nutritional conditions provided by the two very different soil types are also probably involved. Collectively, these observations indicate very limited potential for reducing GNB by controlling insects more rigidly.

Poor harvesting practices can lead to considerable increases in bacterial numbers and endotoxin contamination of fiber. Many studies have shown that concentrations of live bacteria and especially of endotoxin increase with prolonged weathering (exposure to dew and rain) of open bolls (Morey et al. 1983, Simpson et al. 1983, Fischer and Sasser 1987, Simpson et al. 1987a, Reyes et al. 1988, Fischer and Foarde 1989). Bacterial numbers and endotoxin also increase severalfold following frost or freezing damage (Morey et al. 1983, Berni et al. 1988). The impacts of weathering and frost are also seen in correlations between increasing bacterial numbers and decreasing cotton fiber grades (Morey et al. 1980, Simpson and Marsh 1982, Fischer and Sasser 1987) or increasing percentages of immature fibers (Morey et el. 1980, Simpson and Marsh 1982).

Numbers of colony-forming units of live bacteria also may be greater in machine-harvested cotton than in hand-harvested cotton (Simpson et al. 1987c, Fischer and Foarde 1989) and in ginned fiber

than in unginned fiber (Fischer and Foarde 1989). Part of the effect of machine harvesting is probably due to the greater incorporation of trash, which has a higher bacterial titer. However, both mechanical operations probably break up the encrusted bacterial colonies into smaller pieces and may produce more colony-forming units without any real increase in bacterial mass.

Chemical defoliation was shown to increase bacterial numbers on fiber in Texas (table 30) and Mississippi (Reyes et al. 1988). However, chemical defoliation does not cause an increase in bacterial numbers as great as that caused by defoliation from frost (Fischer and Foarde 1989). The effects of defoliation probably involve both the leakage of nutrients from the treated leaves and bracts onto fiber of opened bolls and the stimulation of boll opening to expose immature fibers that can support greater bacterial growth.

Harvesting and other cultural practices should be designed to minimize weathering and percentages of immature fibers in the harvested crop. Defoliants should not be applied until 60 percent or more of the bolls are open. Ideally, weather conditions should be dry after a defoliant is applied so that nutrients leaked from the dying leaves and bracts are not transported to the fibers. Harvesting should be done as soon as possible after the bolls are open and before considerable regrowth occurs. In areas where cotton is harvested after frost, it is important to plant early and use adapted cultivars that will mature most of their bolls before the first freeze occurs. The use of narrow row plantings also may facilitate maturity. Most of these practices are already recommended to achieve maximum yields and grades of fiber and thus are not unique to minimizing bacterial contamination.

Biological Control

Various bacterial pathogens and ice-nucleating bacteria on plants have been controlled by sprays of other bacterial species or strains (Blakeman and Fokkema 1982, Windels and Lindow 1985). Microbial sprays and seed treatments also have been used to control fungal pathogens and insects of cotton. *Bacillus thuringiensis* is routinely used to control *Heliothus* species of insects on cotton, and a mixture of *Bacillus cereus* and *B. megaterium* has been used to suppress the boll weevil (Chang et al. 1988). Commercial preparations of *B. subtilis* and *P. fluorescens* are sold to stimulate growth of cotton, and *P. fluorescens* also has been shown to be antagonistic to the fungal pathogens *Rhizoctonia solani* and *Pythium ultimum* (Howell 1981). Yeasts

Table 30. Concentrations of GNB on seed cotton from bolls treated with various biocontrol agents*

Treatment	Millions of GNB/g			Percentage of GNB		
	E/A	L/A	L/B	E/A	L/A	L/B
Control	0.6	1.4	0.7	18†	42	50
B. thuringiensis	0.7	1.4	0.8	22	43	59
B. subtilis	1.2	1.8	0.6†	30†	44	43
P. fluorescens	1.0	1.6	0.8	22	38	49
S. cerviseae	1.0	1.7	0.8	24†	40	57
Mean	0.9‡	1.6	0.7‡	23‡	42	52†

*Early (E) bolls set June 28, 1988; late (L) bolls set July 11. Bolls harvested 1 day before (B) or 13 days after (A) chemical defoliation on September 13, 1988.

†,‡ Numbers significantly different from those for L/A within the same treatment at the 0.05 and 0.01 percent level, respectively.

Source: Bell et al. (1990).

and bacteria have been used to decrease "stickiness" of cotton fiber, presumably by using sugars and dextrans on the fiber (Balasubramanya et al. 1985). Bell et al. (1990) evaluated the effects of sprays with *B. cereus*, *B. megaterium*, *B. subtilis*, *B. thuringiensis*, *P. fluorescens*, and *Saccharomyces cerviseae* (baker's yeast) on populations of bacteria on seed cotton and fiber for 2 yr at two locations in Texas. Part of these results are shown in table 30. None of the treatments had any significant (p=0.05) effect on numbers of total or GNB, percentages of GNB, or yield of cotton.

The results of the biocontrol treatments indicate that *E. agglomerans* and *P. syringae*, which are predominant on aerial surfaces of nearly all plant species (Dickinson and Preece 1976, Windels and Lindow 1985), are much better competitors under cotton field conditions than any of the biocontrol agents tested. In laboratory studies, Fischer and Foarde (1991b) also found that *E. agglomerans*, *P. syringae*, and *F. oryzihabitans* were more competitive than *B. subtilis* at temperatures of 25 to 35 °C; only at 40 °C and above was *B. subtilis* able to displace the gram-negative species. *Enterobacter agglomerans* was the most competitive of the four species and could displace any of the other three species at 35 °C.

The potential for successful biocontrol is further severely limited by the rapid growth of bacteria on cotton fiber immediately after boll opening, with maximum populations often accumulating within 1 wk of boll opening (Simpson et al. 1987a, Reyes et al. 1988, Fischer et al. 1989b, Heintz et al. 1989, Zuberer and Kenerley 1991). Thus, the crop would need to be sprayed every 4 to 5 days with biocontrol agents during the entire period of boll opening. At this time, it is difficult to get ground equipment into the field and to get good coverage of the bolls with any equipment. If successful biocontrol of endotoxin-producing strains of *E. agglomerans* and *P. syringae* is to be achieved, it probably will be possible only with endotoxin-deficient strains of the same species applied early in the growing season. Control of ice-nucleation-active strains of these two species has been achieved only with non-ice-nucleation-active strains of the same species or with unidentified *Pseudomonas* species (Lindow 1988).

Problems in Studying Bacteria on Cotton

Studies of the ecology, epidemiology, and control of saprophytic bacteria on cotton fiber have been complicated by the following factors: (1) there is a very large inherent error in viable bacterial counts on any plant tissue, (2) methods and materials used to measure bacteria on cotton fiber have been derived largely from medical microbiology and often are not applicable without modification for epiphytic bacteria of plants, (3) selective media for GNB frequently are not completely selective and need to be confirmed by carefully used gram stains, and (4) the effects of moisture and nitrogen availability on bacterial multiplication and survival often complicate studies of other variables.

Statistical evaluation of data is extremely critical in any study of ecology, epidemiology, or control of target pests. Field design, replication numbers, and data transformations can affect the reliability of statistical analyses. Bell and Tribble (1988, 1989) found that significant differences in bacterial densities occurred among different areas of the same apparently uniform field. Thus, some type of design, such as randomized complete blocks replicated four or more times, is needed to detect and compensate for differences among fields or parts of fields.

Bacterial counts also vary greatly among individual locks or bolls within a treatment plot. It is common to find a few bolls that number 100 to 1,000 times greater than the median, and it is impossible to identify visually all of the bolls that will show these unusually high numbers. Consequently, large numbers of samples must be used even to distinguish twofold and threefold differences in bacterial counts among treatments. Much of the variation among samples can be eliminated by blending all of the fiber from a treatment and then combining 10 or more subsamples taken randomly to make up each sample. But even with this approach, a total of 40 or more samples per treatment may be necessary to distinguish twofold differences between treatments (Bell et al. 1987). When random whole bolls are used as samples, 100 or more per treatment may be necessary to detect twofold differences. Variation among bolls can be reduced by tagging open flowers and by harvesting and comparing only bolls that were set on the same date (Bell et al. 1990).

Epiphytic bacteria occur in a lognormal distribution on the phylloplane of plants. Therefore, counts of bacteria must be logarithmically transformed before performing any statistical analysis, if valid conclusions are to be reached. Likewise, data on percentages of bacteria within a class should be arcsine transformed before they are statistically analyzed. The absence of proper field designs, replication numbers, or statistical evaluations makes it difficult to interpret many studies on ecology, epidemiology, and control of gram-negative epiphytic bacteria on cotton.

Studies on the percentage distributions of different bacterial species on cotton fibers have been complicated by variations in incubation temperatures and the use of commercial test kits that are common in medical microbiology. For example, bacteria from cotton often have been grown and tested biochemically at 37 °C, the temperature of the human body. Unfortunately, some of the plant bacteria, especially strains of *P. syringae*, are better adapted to lower temperatures and either do not grow at 37 °C or are under enough stress to cause false negative biochemical results. Bacteria from cotton should be grown and tested at temperatures around 25 to 30 °C. Identifications also have been based on commercial kits and computer programs developed for medical microbiology, and some of these programs do not include common plant bacteria such as *X. campestris*, *P. syringae*, and *E. caratovora* in their database. Thus, these plant bacteria are incorrectly identified as other species. This deficiency in the database has been corrected in some of the recent programs. Many of the studies prior to the mid-1980's, however, underestimated the importance of *P. syringae* because of the high incubation temperatures and the inadequate databases that were used.

GNB are generally counted on media that putatively allow growth of only this type of bacterium. With some of the media used in early studies, there never was any test to verify specificity for bacteria from cotton. In recent years most counts of GNB have been made on trypticase-soy agar amended with cycloheximide to suppress fungi and 15 mg/L of vancomycin to suppress gram-positive bacteria. This medium appears to be well suited for bacteria from cotton, except that some *Clavibacter* species that are gram-positive also grow on the medium. Increasing the vancomycin concentration can correct this problem (Fischer et al. 1992). However, bacteria on any medium should be checked with gram stains to verify the selectivity of the medium before it is used in critical studies. The best methods for identifying and enumerating bacteria on cotton fiber have been reviewed (Fischer et al. 1992).

In spite of the inadequacies in methods and materials in many studies of ecology, epidemiology, and control of saprophytic GNB on cotton, it is obvious that very little progress can be made in controlling populations of these bacteria in the field beyond what is already achieved by using recommended procedures for producing high-quality cotton fiber. Most of the recommendations for controlling boll rots (Cauquil 1975, Roncadori et al. 1975) and bacterial blight (Davis and Sandidge 1977, Watkins 1981) are equally applicable, but less effective, for reducing growth of saprophytic bacteria on cotton. These recommendations include the use of cultivars that are adapted to the area and known for producing high grades of lint there, minimum use of irrigation and nitrogen fertilization that is consistent with economic yields, insect control to minimize boll injury, application of chemical defoliants only after 60 percent or more of the bolls are open (preferably prior to dry weather), and prompt harvest before regrowth or frost. The major objective is to minimize the inoculation and harvest of immature fibers, which have very high concentrations of bacteria. No single factor will reduce GNB numbers by more than 50 to 75 percent. When used together, these practices probably can reduce the population by 90 percent or more. However, even the best combination of controls will still allow growth of more than 0.1 million GNB per gram of fiber in moist, humid climates. Consequently, additional procedures remain necessary to control bacterial numbers in the air of cotton mills.

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Chapter 9. Potentially Byssinogenic Bacteria That Grow on Raw Cotton Fiber During Weathering in the Field

Marion E. Simpson and Paul B. Marsh

Prominent among the gram-negative bacteria (GNB) on cotton fiber are *Pseudomonas syringae* and *Enterobacter agglomerans*, the latter known in the botanical literature as *Erwinia herbicola*. These bacteria also grow frequently as epiphytes on the leaves of many plants other than cotton, often producing no visible symptoms in the leaves but under some circumstances causing necrosis and severe damage to any of several important crop plants. They are especially adapted to growth on plants in aboveground situations. GNB in the dust-laden air around grain elevators containing wheat are reported to be very similar in identity to those in cotton dust, suggesting a possible basic similarity in the cause of pulmonary difficulties from both types of dusts. In parallel with the cotton fiber data, seeds of rice and several other important crop plants are also reported to have *Erwinia herbicola* on them.

Our purposes here are (1) to summarize the results of investigations of the numbers and kinds of GNB observed to occur as part of the normal flora on raw cotton fiber weathered in the field before harvest and to delineate methods of identifying members of this bacterial population, (2) to indicate the circumstances under which such bacteria grow on cotton fibers and on aboveground parts from other plants (including seeds), and (3) to briefly describe some currently applicable practical implications of these findings and at the same time provide enough detail to be useful to investigators wishing to undertake further research in the area.

Natural Occurrence of Bacteria on Raw Cotton Fibers

The findings of past research are reviewed here and include the following major themes: (1) the gram-negative bacterial population on raw cotton fiber consists mainly of a small number of bacterial species, frequently present as large numbers of bacterial cells and often including *Enterobacter agglomerans* or *Pseudomonas syringae* or both, (2) the bacteria grow on the fiber mainly during exposure to wet weather in the field before harvest, (3) these bacteria are dispersed from the fiber into the air of the mill during mechanical fiber processing and are frequently present in higher concentrations in the air of a mill than in other situations, and (4) many of the same cotton fiber-inhabiting bacteria grow in the field on senescent aboveground parts of other plants in addition to cotton.

Clark et al. (1947) reported that the bacterial population of cotton fiber from bolls which had opened prematurely under the influence of frost in northern Texas and Oklahoma consisted of extremely large numbers of cells of *Aerobacter cloacae* (= *Enterobacter cloacae*). The fiber was thin-walled and unsuited for spinning and was used in the manufacture of mattresses. Some of the workers handling the fiber experienced severe difficulty in breathing, along with conjunctival irritation, generalized aches, fatigue, cough, chills, fever, nausea, and vomiting (Neal et al. 1942, Schneiter et al. 1942, Caminita et al. 1943, Clark et al. 1947). The authors noted that the bacteria in the soil in which the plants had grown constituted a widely diverse group of many species, of which *Enterobacter cloacae* was not a dominant member. A brief history of *E. cloacae* is provided by Edwards and Ewing (1972).

Pernis et al. (1961) showed that endotoxins of bacterial origin were present in dusts in the air of some textile mills. The concept that dust dispersed from cotton fiber and inhaled by workers can cause breathing difficulty because of the presence of endotoxin-bearing GNB is a long-standing idea detailed and reviewed with much supporting data in several publications (Cavagna et al. 1969, DeMaria and Burrell 1980, Olenchock et al. 1985, Berni et al. 1988, DeLucca et al. 1988, Rooke 1989). Crosse (1971) noted that earlier work had shown that *Erwinia herbicola* (*Enterobacter agglomerans*) is commonly dominant in the epiphytic flora of many plants. *Enterobacter agglomerans* is, therefore, readily accessible to grow on cotton plants in the field.

Rylander and Lundholm (1978) published results of a major study in which they detected certain bacteria on raw cotton fiber and identified several of them. They reported 1,300 to 1.3 million gram-negative rods per gram of fiber for 27 samples from the United States, Russia, and other countries. They

found *Enterobacter agglomerans* [also known in the botanical literature as *Erwinia herbicola* (Perombelon 1981)] and *Pseudomonas syringae* on almost all samples and *Agrobacterium* spp. on about 60 percent of the samples. *Enterobacter agglomerans* is described by Ewing and Fife (1972).

Fischer et al. (1979) reported that GNB can be found in association with leaf trash in raw cotton, including fragments of weeds present in the field where the cotton was grown. Also, Rylander et al. (1979) reported a range of 250 to 5 million GNB per gram on fiber being processed among 23 different U.S. cotton mills. Schaad (1980) identified *Pseudomonas syringae* as the cause of disease in tobacco, peas, snapdragon, delphinium, tomato, beans, peaches, and other plants. Therefore, *Pseudomonas syringae* is commonly present on a wide variety of plants as part of the GNB.

Millner et al. (1982) demonstrated the presence of *Enterobacter agglomerans*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, and *Enterobacter sakazakii* on samples of commercial raw fiber grown in the southeastern United States, and Millner et al. (1984) presented further identifications of GNB on raw cotton. They found no *Pseudomonas syringae* but found large numbers of an unknown *Pseudomonas*.

Millner et al. (1983) showed that bacteria in the air of a cotton mill could sometimes rise to a level of approximately 10,000 bacterial cells per cubic meter, whereas the level in the air of a "clean room" was about 100. Clearly, the number of bacteria present per unit volume in mill air was much higher than in most other situations to which individuals are usually exposed in their occupations or daily living experiences. In many other circumstances, the total bacterial level is usually below 500/m^3 (Greene et al. 1962).

Simpson and Marsh (1982) reported a range from 10,000 to 290 million bacteria per gram of fiber in 143 commercial samples of cotton fiber from the U.S. crop of 1980. Simpson et al. (1983) showed that fiber from unopened, undamaged, nearly mature field-grown bolls was initially sterile (table 31) but

Table 31. Results of tests for bacteria on unopened, unpunctured bolls

Origin of bolls	Date received	Number tested	Number of bolls with bacterial outgrowth on: TSA medium*	TSA medium plus vancomycin
Auburn, AL	9/23	10	0	0
College Station, TX	9/15	10†	0	0
Florence, SC	9/11	10	0	0
Lubbock, TX	9/23	10	0	0
Phoenix, AZ	8/20	8	0	0
Shafter, CA	8/21	6	0	0
Stoneville, MS	8/19	2	0	0
Stoneville, MS	8/27	13	0	0
Stoneville, MS	9/8	10	1‡	1‡
Stoneville, MS	9/10	10	0	0

*TSA medium is a trypticase soy agar.
†Four of the bolls had a reddish-brown discoloration at the base, which is indicative of senescence of the tissues. Bacterial movement through these tissues to the fiber was not detected.
‡Boll with an undetected insect puncture.

Source: Simpson et al. (1983).

Table 32. Aqueous extract pH, DNS (dinitrosalicylic acid) value, and bacteria level in fiber*

Location	Weeks of weathering	Fiber pH†	DNS value‡	Millions of bacteria/gram of fiber		
				Total	GNB	Presumptive coliform
Raleigh, NC	0	6.3	.19	0.13	0.04900	0.00020
	2	6.8	.07	0.79	0.13000	0.00490
	5	9.4	.00	1.30	0.03300	0.01300
	10	8.3	.14	1.70	0.01700	0.00020
Florence, SC	0	6.1	.30	0.049	0.03100	0.00020
	2	6.6	.20	1.100	0.00170	0.04900
	5	9.4	.01	0.490	0.04900	0.00170
	8	9.4	.00	0.700	0.11000	0.00230
Auburn, AL	0	6.3	.36	0.033	0.02300	0.00700
	2	6.9	.08	0.330	0.23000	0.01300
	5	7.4	.03	1.300	0.13000	0.00080
	10	7.4	.02	0.790	0.03300	0.00020
Stoneville, MS	0	6.2	.25	0.002	0.00020	0.00020
	2	6.5	.19	0.490	0.13000	0.02300
	5	8.3	.02	1.300	0.02300	0.00020
	10	7.7	.00	1.300	0.02300	0.00020
Lubbock, TX	0	6.0	.34	0.002	0.00130	0.00020
	2	6.3	.24	0.049	0.02300	0.00270
	5	8.4	.08	2.600	5.40000	1.70000
	10	8.3	.06	0.230	0.13000	0.00490
Phoenix, AZ	0	6.3	.41	1.300	0.54000	0.54000
	2	6.3	.18	0.070	0.00490	0.00230
	5	6.5	.24	0.330	0.17000	0.00790
	10	6.4	.30	0.130	0.07900	0.04900
Shafter, CA	0	6.4	.16	0.002	0.00004	0.00002
	2	6.4	.33	0.002	0.00020	0.00020
	5	6.2	.52	0.017	0.00020	0.00020
	10	6.3	.19	0.005	0.00020	0.00020

*The plants from which the fiber was collected were grown in field plots across the U.S. Cotton Belt in 1980. All bolls were well fluffed prior to weathering 0–10 wk.

†Higher values result from microbial decomposition of organic acids in fiber (Marsh et al. 1951, Marsh and Simpson 1986).

‡Lower values result from microbial use of reducing sugars in fiber (Marsh et al. 1958).

Source: Simpson et al. (1983).

Table 33. DNS values and bacterial counts of four cotton genotypes grown at College Station, TX, in 1980*

Cotton varieties	Weeks of weathering	DNS value	Millions of bacteria/gram of fiber†		
			Total	GNB	Presumptive coliform
Coker-420	0	.13	0.022	0.00079	0.00002
	2	.17	1.700	0.79000	0.13000
	5	.02	11.000	0.70000	0.01100
	10	.03	17.000	0.33000	0.07900
Deltapine 16	0	.06	0.130	0.00027	0.00002
	2	.10	2.300	0.07000	0.02300
	5	.03	160.000	0.79000	0.33000
	10	.06	22.000	0.23000	0.00400
Stoneville 213	0	.19	0.007	0.01130	0.00020
	2	.29	7.000	0.49000	0.04900
	5	.02	17.200	0.79000	0.13000
	10	.07	33.000	3.30000	0.01100
Tamcot SP–37	0	.26	0.070	0.00790	0.00013
	2	.32	3.400	0.46000	0.04600
	5	.01	10.000	0.33000	0.00490
	10	.02	23.000	7.90000	0.13000

*All bolls were well fluffed prior to weathering for 0–10 wk.
†No differences among the varieties were detected.

Source: Simpson et al. (1983).

that following normal opening of the bolls and good fluffing of the fiber, the bacterial content of the fiber during exposure to wet weather and before harvest could increase to a level as high as 2.6 million bacterial cells per gram of lint. Fiber subjected to dry climate weathering at Shafter, CA, however, maintained very low bacterial levels (see data near bottom of table 32).

Cotton fiber in bolls exposed to wet weather during boll opening is especially prone to microbial problems. In some of these bolls, especially bolls in deep shade under cotton leaves, the fiber may be delayed in its initial drying and may never fluff properly (Marsh et al. 1950). Simpson et al. (1983) showed that such fiber may become massively infected with both fungi and bacteria, with occasional levels of bacteria as high as 24 billion per gram of fiber. The term "microbial tightlock" has been used to designate the poorly fluffed condition (Marsh et al. 1950). Marsh et al. (1985) suggested that microbial tightlocks, with their very high level of microbes, might be important in causing byssinosis.

Simpson et al. (1983) found increases in the levels of total bacteria and GNB during preharvest weathering of four cotton varieties at College Station, TX, and decreases in DNS (dinitrosalicylic acid) values; however, the bacterial counts increased more rapidly than the DNS values decreased (table 33). The period from boll opening to harvest is variable but often exceeds 8–10 wk for early maturing bolls.

Morey et al. (1983) reported that GNB are present on bracts, pericarps, and leaves of cotton. Also, Hirano and Upper (1983) reviewed numerous reports indicating that *Pseudomonas syringae* may occur in large numbers as a resident on symptomless leaves of various plants. However in some of

Table 34. Date of harvest, weeks of weathering, rainfall since previous harvest, fiber pH, and bacterial counts for fiber from snapped bolls at Raleigh, NC, in 1985

Date of harvest	Weeks of weathering	Rainfall in period (mm)	Fiber pH	Millions of bacteria/gram of fiber		
				Total	GNB	Presumptive coliform
9/9/85	0	-	6.5	0.098	0.0029	0.000047
9/16/85	1	0.0	7.6	1.800	0.0200	0.000030
9/23/85	2	5.7	6.5	0.150	0.0029	0.000047
9/30/85	3	1.8	7.2	0.410	0.0160	0.000045
10/7/85	4	27.2	7.8	5.300	0.0430	0.000016
10/14/85	5	0.0	7.2	2.200	0.0500	0.000043
11/9/85	8–10	88.0	9.0	4.600	0.0130	0.003700

Source: Simpson et al. (1987a).

these plants, *P. syringae* may also cause necrotic damage and plant injury under suitable conditions. Leben (1981) reviewed the general subject in detail.

Simpson and Marsh (1984) reported that for the 1981 crop, GNB were found at a million or more per gram of commercial raw cotton fiber in samples from the Southeast, Midsouth, Texas, and Oklahoma. Also, Simpson et al. (1984) reported that in the 1982 crop in these areas, bacteria in commercial fiber varied greatly in number and the number had little effect on grade. Gram-negative counts of a million or more per gram of fiber occurred widely and sporadically.

Simpson and Marsh (1985) presented information on bacterial counts in California on cottons from the crop of 1983. Low counts were found in samples from the San Joaquin Valley, an area that is typically dry between boll opening and harvest.

Simpson and Marsh (1986) reported counts on commercial fiber from across the Cotton Belt in the crop of 1984. Many more samples with high bacterial counts were encountered in 1984 than in prior years, and these high counts were attributed to the large numbers of rainy days between boll opening and harvest in 1984. Simpson et al. (1986) reported that in 1980 and again in 1983 the bacterial counts in fiber from newly opened fluffy cotton bolls increased during rainy periods of weathering.

Marsh and Simpson (1987) reported viable counts for total, gram-negative, and coliform bacteria on fiber from across the Cotton Belt in the crop of 1985. As in earlier work, the counts from the 1985 crop were highly variable even within a single grade from a single location. No clear relation of counts to grade was seen. Many samples, however, had a total count of more than 10 million per gram of fiber and many had more than a million GNB per gram. Average levels for the crops of 1980–1984 were presented. Samples from the San Joaquin Valley of California had consistently lower counts than the beltwide average, a result attributed to dry weather there between boll cracking and harvest.

Simpson et al. (1987a) reported bacterial counts on fiber from cotton bolls weathered for 0 to 10 wk at Raleigh, NC (table 34), Auburn, AL (table 35), and Stoneville, MS (table 36). Decreased numbers of viable cells during the weathering periods were attibuted to desiccation and to exposure to ultraviolet light. Dead GNB and their endotoxins were still present but were not counted.

Simpson et al. (1987c) reported bacterial counts on fiber of five cotton varieties weathered in the field at Stoneville, MS, but found no major differences in counts linked with either variety or method of picking, whether by hand or machine. Simpson et al. (1987b) reported bacterial counts on fiber col-

Table 35. Date of harvest, weeks of weathering, rainfall since previous harvest, fiber pH, and bacterial counts for fiber from snapped bolls at Auburn, AL, in 1985

Date of harvest	Weeks of weathering	Rainfall in period (mm)	Fiber pH	Millions of bacteria/gram of fiber		
				Total	GNB	Presumptive coliform
8/26/85	0	-	6.6	0.20	0.012	0.000047
9/3/85	1	21.0	7.0	14.00	3.000	0.000110
9/10/85	2	3.0	8.3	74.00	9.200	0.000440
9/16/85	3	0.0	8.9	0.65	0.053	0.000097
9/24/85	4	20.5	9.0	1.70	0.180	0.000170
10/1/85	5	16.2	7.9	29.00	4.100	0.003200
10/14/85	8–10	9.0	7.3	6.40	0.680	0.000320

Source: Simpson et al. (1987a).

Table 36. Date of harvest, weeks of weathering, rainfall since previous harvest, fiber pH, and bacterial counts for fiber harvested after specified weathering periods at Stoneville, MS, in 1985

Date of harvest	Weeks of weathering	Rainfall in period (mm)	Millions of bacteria/gram of fiber			
			Fiber pH	Total	GNB	Presumptive coliform
8/26/85	0	-	6.9	0.28	0.0100	0.000047
9/2/85	1	0.5	7.0	4.50	0.0990	0.000047
9/9/85	2	60.8	9.5	11.00	0.4300	0.000039
9/16/85	3	1.5	8.9	4.00	0.0250	0.000100
9/23/85	4	1.0	9.2	0.42	0.0057	0.000047
10/1/85	5	22.8	8.0	5.90	0.2600	0.000093
11/17/85	10	15.90	8.5	19.00	0.1300	0.001100

Source: Simpson et al. (1987a).

lected on three dates from well-fluffed locks of seven varieties at two locations near College Station, TX. Consistently higher counts were obtained on fiber from one of the two locations, but no consistent varietal differences were seen.

Simpson and Marsh (1987) summarized bacterial counts on cotton from the crops of 1980–1984. Total counts were extremely variable but often very high, in the range of 1 to several million per gram of fiber. Counts on fiber from the San Joaquin Valley of California, an area usually very dry in the period between boll cracking and harvest, were regularly very low.

Fischer and Sasser (1987) observed *Enterobacter agglomerans* and *Pseudomonas syringae* on raw cotton grown in Louisiana. Baca and Moore (1987) found large numbers of *Pseudomonas syringae* on symptomless roadside grasses. More than half of their isolates were pathogenic on tomato. Kremer (1987) reported the presence of *Erwinia herbicola* on seeds of certain weeds.

Table 37. Numbers of isolates of various bacteria isolated from fiber from individual cotton bolls grown at Florence, SC, in 1986

Bacterial species	Number of isolates on fiber*		
	Unweathered bolls		Tightlock bolls
	Normal	Damaged	
Escherichia coli	0	0	0
Enterobacter agglomerans	4	16	18
Enterobacter cloacae	0	1	2
Pseudomonas aeruginosa	0	0	0
Pseudomonas fluorescens	0	0	0
Pseudomonas putida	0	3	2
Pseudomonas syringae-viridiflava	3	11	4
Pseudomonas "A"	1	1	3
Pseudomonas "O"	8	6	6
VE–1	0	0	1
VE–2	16	18	9
Not identified	5	11	6

*GNB identifications based on *Bergey's Manual of Determinative Bacteriology* and *Bergey's Manual of Systematic Bacteriology* (Buchanan and Gibbons 1974, Krieg and Holt 1984).

Source: Simpson et al. (1988).

Lacey and Lacey (1987) reported finding mainly *Enterobacter agglomerans* in the air of English cotton mills, but also *Enterobacter cloacae*, *Flavobacterium* spp., *Acinetobacter calcoaceticus*, *Alcaligenes* spp., *Pseudomonas* spp. and cocci. They found total bacterial counts up to 1.5×10^5 colony forming units per cubic meter in one mill and up to 2.3×10^5 in the air of a second mill.

Simpson et al. (1988) recorded results of observations in which they had observed the presence of *Enterobacter agglomerans*, *Pseudomonas syringae*, a *Pseudomonas*-like organism designated as "VE–2," and other bacterial isolates on field-weathered raw cotton collected from plants near Florence, SC (table 37), and College Station, TX (tables 38 and 39). The VE–2 bacterium has been identified as *Flavimonas oryzihabitans* (Holmes et al. 1987). The identification methods used by Simpson et al. (1988) were clearly capable of identifying many other bacteria, and, in fact, minor amounts of several others were detected and recorded. Interestingly, *Xanthomonas malvacearum*, the cause of the common bacterial boll rot and angular leaf spot of cotton, was not detected.

Simpson and Marsh (1988) presented bacterial counts on commercial cotton fiber from the crop of 1986. Data averages showed that the levels for total bacteria and gram-negatives were distinctly higher for the 1986 crop than for the crops of 1984 and 1985.

Spear et al. (1988) exposed guinea pigs to an aerosol consisting of ground cellulose powder plus cells of *Enterobacter agglomerans* and observed a half dozen characteristic pulmonary responses. They also reported the same or very similar responses after exposure to a natural cotton dust.

Akinwunmi et al. (1989) reported finding *Enterobacter agglomerans*, *Pseudomonas syringae*, *Pseudomonas* VE–2, *Pseudomonas denitrificans*, and *Xanthomonas campestris* on leaves, flowers, lint, bracts, and seeds of cotton plants.

Table 38. Number of isolates of various bacteria isolated from fiber from individual cotton bolls grown at College Station, TX, in 1986 and collected during a first harvest

Bacterial species	Number of isolates from fiber			
	Upland field		Bottomland field	
	Bottom*	Top*	Bottom*	Top*
Escherichia coli	2	2	0	0
Enterobacter agglomerans	18	41	46	29
Enterobacter cloacae	3	2	7	5
Enterobacter intermedium	0	1	0	0
Pseudomonas aeruginosa	2	0	2	0
Pseudomonas fluorescens	1	0	0	0
Pseudomonas putida	9	5	6	0
Pseudomonas syringae	49	30	13	24
Pseudomonas viridiflava	0	0	0	0
Pseudomonas "A"	9	4	9	8
Pseudomonas "O"	2	3	3	5
VE–1	0	0	5	4
VE–2	23	24	31	33
Not identified	6	1	16	2

*Bottom or top of cotton plant.
Source: Simpson et al. (1988).

Simpson et al. (1989) reported the identification of 2,990 gram-negative isolates from 702 classers' samples from the crop of 1987 in the Texas-Oklahoma and western cotton regions (table 40). *Enterobacter agglomerans*, *Enterobacter cloacae*, *Pseudomonas putida*, *Pseudomonas syringae*, and members of the *Pseudomonas* VE group were the most frequently encountered. This high frequency of occurrence does not, of course, indicate that these organisms have the highest potency per cell in inciting byssinotic symptoms.

Simpson et al. (1990) reviewed the kinds of potentially byssinogenic GNB that are found on cotton fiber.

Discussion

Data from our laboratory are described in several original publications. Methods for viable bacterial counts (Simpson and Marsh 1982) and for bacterial species identifications (Simpson et al. 1989) have been detailed and are mentioned here but not redescribed here. The basic rationale and methodology of a procedure for aqueous-extract pH of raw cotton fiber have been presented (Marsh et al. 1951, Marsh and Simpson 1986). Elevated pH levels result from microbial decomposition of organic acids in the fiber and reflect a fiber history of exposure to wet weather. Low values in a method for fiber glucose, the dinitrosalicylic acid (DNS) procedure, also reflect microbial action during exposure of fiber to wet weather (Marsh et al. 1958).

From the studies and data mentioned here (for example, Simpson and Marsh 1982, 1984, 1986, and 1988 and in tables 32 through 36), one can conclude that many bales of cotton fiber have so many bacteria in them that the input of bacteria into a carding machine is often very high. Only a small percentage of these bacteria would have to be dislodged during the carding process to accomplish movement of a great many bacteria into mill air.

Table 39. Number of isolates of various bacteria isolated from fiber from individual cotton bolls grown at College Station, TX, in 1986 and collected during a second harvest

Bacterial species	Number of isolates from fiber			
	Upland field		Bottomland field	
	Bottom*	Top*	Bottom*	Top*
Escherichia coli	1	0	0	0
Enterobacter agglomerans	10	18	16	16
Enterobacter cloacae	3	1	3	2
Enterobacter intermedium	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0
Pseudomonas fluorescens	0	0	0	0
Pseudomonas putida	4	3	4	0
Pseudomonas syringae	15	28	2	3
Pseudomonas viridiflava	0	0	0	0
Pseudomonas "A"	18	16	22	20
Pseudomonas "O"	6	1	10	4
VE–1	1	0	1	0
VE–2	18	23	21	24
Not identified	27	17	10	8

*Bottom or top of cotton plant.

Source: Simpson et al. (1988).

Thus, for example, for cotton with a million bacteria per gram of fiber, this corresponds to 454 million bacteria per pound. A card using this cotton and operating at 1 lb of fiber per minute would take in almost a billion bacteria in 2 min. Dislodgment and release into air of only 1 percent of these bacteria at the card would involve transfer into the air of about 10 million bacteria in 2 min. Common environmental background levels of bacteria in air is in the general range of a few hundred to a very few thousand per cubic meter (Bourdillon et al. 1949, Colebrook and Cawston 1949, Williams et al. 1956, Greene et al. 1962, Andersson et al. 1973, Rosler 1976); therefore it is likely that bacterial levels of air around a card are much higher than background levels.

In the case of unexpected or unusually severe episodes of acute byssinosis in a cotton mill, it might be of interest to determine whether the kinds of GNB present included some types beyond the commonly present types named here and whether gram-positive bacteria could be the cause. Efforts to identify the bacteria that cause byssinosis have focused on GNB, but it seems uncertain whether gram-positive bacteria could also be involved. The basic causative agent has been assumed by many investigators to be endotoxins, and endotoxins are known to be produced by GNB. Since there are no sharply diagnostic symptoms of acute endotoxin exposure, however, it is impossible to tell whether byssinosis is due to endotoxin exposure or some other factors such as exposure to gram-positive bacteria.

The strongly limited range of different species of GNB reported here on preharvest-weathered cotton fiber parallels a previous finding that the range of types of saprophytic fungi on the seeds of various crop plants is similarly very limited. Among the commonly occurring fungi entering a cotton mill on the fiber are *Alternaria* sp. and *Cladosporium herbarum*. Both fungi are known to cause asthma in susceptible individuals with broncho-constrictive

Table 40. Number of samples and isolates and number and percentage of the most commonly isolated bacterial species in the cotton crop of 1987

Location	Total number of: Samples	Isolates	Number (and percentage)* of isolates for species in: *Enterobacter agglomerans*	*Enterobacter cloacae*	*Pseudomonas putida*	*Pseudomonas syringae*	VE Group†
Texas-Oklahoma Region							
Abilene, TX	74	337	195 (58)	38 (11)	17 (05)	31 (09)	31 (09)
Altus, OK	33	184	97 (53)	15 (08)	10 (05)	23 (12)	16 (09)
Corpus Christi, TX	40	223	57 (26)	21 (09)	12 (05)	31 (14)	24 (11)
El Paso, TX	60	297	133 (45)	31 (10)	16 (05)	44 (15)	35 (12)
Harlingen, TX	54	251	56 (22)	11 (04)	18 (07)	18 (07)	30 (12)
Lamesa, TX	56	263	131 (50)	29 (11)	17 (06)	34 (13)	26 (10)
Lubbock, TX	42	212	121 (57)	8 (04)	24 (11)	19 (09)	9 (04)
Waco, TX	57	283	90 (32)	12 (04)	17 (06)	42 (15)	40 (14)
Western Region							
Bakersfield, CA	66	139	53 (38)	14 (10)	6 (04)	19 (14)	9 (06)
El Centro, CA	69	263	75 (28)	16 (06)	22 (08)	30 (11)	21 (08)
Fresno, CA	73	196	60 (31)	10 (05)	20 (10)	45 (23)	17 (09)
Phoenix, AZ	78	342	105 (31)	28 (08)	15 (04)	75 (22)	52 (15)
All locations	702	2,990	1,173 (39)	233 (8)	194 (6)	411 (14)	310 (10)

*Percentage of isolates of the designated bacterial type from each location.
†VE group contains *Pseudomonas*-like VE–1 and VE–2 organisms (Simpson et al. 1990). VE–1 and VE–2 organisms are not separated here but can be separated on the basis of nitrate reduction.

Source: Simpson et al. (1989).

symptoms similar to byssinosis. A series of papers on the range of GNB on cotton is reviewed in Marsh and Simpson (1982) and Simpson and Marsh (1983). *Enterobacter agglomerans* and *Pseudomonas* spp. were found to be especially prevalent among bacterial isolates from dust collected around wheat elevators (DeLucca et al. 1984). Earlier, James et al. (1946) identified "*Bacterium herbicola aureum,*" known more recently as *Erwinia herbicola* (= *Enterobacter agglomerans*) as a common bacterium observed on stored wheat seeds. Pepper and Kiesling (1963) reported that "*Bacterium herbicola aureum*" was common on the seeds of barley. Otta (1977) isolated *Pseudomonas syringae*, the incitant of leaf necrosis of spring and winter wheat in South Dakota, from diseased leaves of wheat, corn, foxtail, and sorghum and from wheat kernels and could not differentiate this isolate of *P. syringae* from known isolates of *P. syringae* in biochemical, serological, or pathological tests. Similar to those results obtained with cotton dust, these results may indicate that the bacterial flora on various plants (leaves, stems, seeds) depends more on the aboveground environmental situation (that is, changing conditions of moisture and sunlight) than on the plant substrate.

Dutkiewicz (1978) reported that "gram-negative rods proved to be the most common airborne microorganisms in grain stores and mills. The most abundant were bacteria belonging to the species *Erwinia herbicola*—which formed 21.8 to 57.7 percent of the total count." Viable microorganisms were found in numbers up to 1.3 million per cubic

meter of air. DeLucca and Palmgren (1987) reported that *Enterobacter agglomerans* was the predominant bacteria in dusts around grain terminals. Adetuyi et al. (1985) found that *Enterobacter agglomerans* and *Pseudomonas syringae* were common on wheat seeds. DeLucca et al. (1978) found *Enterobacter agglomerans* on rice seeds from Arkansas.

Individuals interested in *Pseudomonas syringae* as a plant pathogen will find information on this organism in publications from the American Phytopathological Society. Topics include the pathogen on sorghum as a cause of bacterial leaf spot (Fredericksen 1986), on corn as a cause of the holcus spot (Shurtleff 1980), on alfalfa as a cause of bacterial stem blight (Graham et al. 1979), on soybean as a cause of bacterial blight (Sinclair and Backman 1989), and on barley (Mathre 1982) and wheat (Wiese 1987) as a cause of leaf blight.

In purchasing cotton for spinning, mills may find it advantageous to avoid, whenever possible, fiber that has been exposed to wet weather before harvest, such exposure being clearly linked to bacterial contamination potentially capable of causing byssinosis. Although pH tests of fiber cannot be used to identify all contaminated samples, a raw fiber sample with a pH of 8.0 or higher can be reliably assumed to have been exposed to wet weather and therefore contaminated.

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Chapter 10. Field Studies of Cotton Grown in the United States

Janet J. Fischer

This chapter summarizes research data from my laboratory at the University of North Carolina over a 15-yr period. The purpose of the studies was to evaluate the microbial flora associated with cotton supplied by various investigators. The methods used in these studies involved counts of viable microorganisms spread on plates with small amounts of serial dilutions of aqueous extracts of cotton. Included in this chapter are summaries (in chronological order) of studies that show the importance of geographical area (and therefore weather conditions) on counts of GNB in cotton.

Variety Studies

In 1974 and 1975, cottons and some plant parts were harvested from many growing areas in the United States. The cotton variety and grade, the method of cultivation, the type of defoliation (by chemicals or by frost), and the method of harvesting for these cottons varied. It was therefore difficult to compare the bacterial counts from one area to another. Two bales were harvested from each area. Table 41 shows GNB counts on two different bales of several varieties from each area in each of 2 yr. The table shows that variability in GNB in cotton occurs between the following:

1. Between two bales of the same variety from the same area in the same year
2. Between bales of the same variety and same area from two different years
3. Between bales harvested from different areas in the United States. (GNB counts in the California area are generally low, and GNB counts in Alabama, Georgia, Louisiana, Mississippi, Arkansas, Texas, and Oklahoma are usually higher.)

After these bales were analyzed for GNB content, they were taken to the model cardroom at the North Carolina State University School of Textiles. The dust levels and counts of airborne GNB were determined during carding. These data are listed in table 42 and show the following:

1. The level of airborne dust liberated in the model cardroom varies between two bales of the same cotton in the same year.

Table 41. GNB in baled cotton

		GNB (cfu/g x 10^{-5})			
		1974		1975	
State	Variety	Bale 1	Bale 2	Bale 1	Bale 2
AL	Coker 310	17	9.7	5.0	--
AR	Deltapine 16	2.0	11	6.7	4.2
CA	Acala SJ3	<0.001	<0.01	0.03	0.006
GA	McNair 511	1.3	0.39	4.7	3.0
GA	Coker 201	6.5	13	63	16
LA	Stoneville 213	2.7	4.8	3.4	3.2
NM	Acala	0.7	0.45	--	0.14
NM	Pima	0.8	0.9	0.09	--
NC	Coker 310	1.0	1.3	22	12
OK	Lockett	12	70	120	23
TN	Coker 310	0.4	0.2	3.0	3.1
TX	Tamcot or Paymaster	0.1	--	3.7	20

2. Dust levels vary from year to year in cotton from one location.
3. Dust levels do not always correlate with counts of airborne GNB.

These studies also showed variability in the counts of gram-positive organisms isolated from cotton and in the numbers of fungi found (data not shown). The results of tables 41 and 42 suggest that growing conditions and area of growth of the cotton might be important in producing cotton that has minimal GNB content and that will liberate minimal amounts of dust and its associated GNB and endotoxin.

Other cotton plant parts besides lint give off dust when cotton is processed. Table 43 compares the counts of GNB in the dust produced from lint, bracts, and leaves from cotton grown in selected areas in 1975. The bracts and leaves were hammermill ground, and determinations were made on the dusts. The bracts and leaves occasionally had high counts of GNB, but, as pointed out by Fischer et al. (1986), the percentage of the baled cotton contributed by the bract and leaf parts was so small that 67 percent of the GNB measured in baled cotton was thought to have come from the lint.

Table 42. Airborne dust and GNB produced from cotton during carding

			Dust (mg/m³)		GNB (cfu/m³)	
State	Variety	Bale	1974	1975	1974	1975
AL	Coker 310	1*	0.542	0.261	3,400	910
AR	Deltapine 16	1	0.299	0.359	2,400	5,200
		2	0.250	0.393	2,450	6,600
CA	Acala SJ3	1	0.375	0.273	1,450	276
		2	0.299	0.299	1,360	180
GA	McNair 511	1	0.391	0.497	1,320	7,000
		2	0.356	0.553	960	3,670
GA	Coker 201	1	0.209	0.347	1,290	2,330
		2	0.144	0.335	340	2,600
LA	Stoneville 213	1	0.371	0.252	2,550	720
		2	0.341	0.208	340	930
MS	Stoneville 213	1	0.340	0.216	60	2,000
		2	0.379	0.285	550	6,600
NM	Acala	1	0.519	0.273	360	120
		2	0.422	0.230	410	130
NM	Pima	1	0.766	0.496	2,500	600
		2	0.551	0.546	3,380	3,400
NC	Coker 310	1	0.301	0.561	840	10,200
		2	0.292	0.414	480	14,000
OK	Lockett	1	0.458	0.274	60	490
		2	0.539	0.277	660	3,200
TN	Coker 310	1	0.251	0.317	120	300
		2	0.286	0.305	280	1,140
TX	Paymaster or Tamcot	1	0.354	0.471	160	1,600
	Paymaster or Tamcot	2	0.304	0.647	170	900

*A second bale was not available from this variety and location.

Table 43. GNB in dust from plant parts harvested in 1975

State	Cotton	GNB (cfu/mg) from: Bracts	Leaves	Lint
AL	Coker 310	0.41	0.20	0.50
CA	Acala SJ3	0.067	0.35	0.0018
GA	McNair 511	1.13	3.53	0.38
GA	Coker 201	0.77	1.31	1.11
LA	Stoneville 213	18.43	33.67	0.325
NM	Acala	0.067	0.094	0.0144
NM	Pima	2.24	0.36	0.009
NC	Coker 310	0.30	0.31	1.70
OK	Lockett	0.089	0.20	7.15
TN	Coker 310	0.05	0.97	0.305
TX	Paymaster	18.0	1.60	1.18

The correlation coefficient of the counts of GNB on raw cotton with the counts of airborne GNB was 0.89, using all data from 1975.

Classers' Samples

Classers' samples are obtained from cotton after it is baled and are used to grade the cotton and establish its market value. They are relatively fresh when obtained and should provide reliable counts when processed promptly.

Cottons from the crops of 1977, 1978, 1979, and 1980 from the five major U.S. growing regions were studied. These cottons were studied in our laboratory and provide an overall view of the variability of the content of GNB in different cottons from different areas and different crop years. Determinations of GNB and endotoxin made on Classers' samples of commercial raw cotton fiber were reported previously (Fischer and Jacobs 1985). A large number of Classers' samples from the 1980 crop showed significant differences in GNB and endotoxin levels among geographical regions (Morey et al. 1984). Area of growth of the cotton was also thought to have an important effect on airborne levels of GNB and endotoxin during carding. Similar studies were conducted by Simpson and Marsh (1982, 1984) on the 1980 and 1981 crops and subsequently on commercial raw cottons from a number of crop years. These studies are outlined in chapter 9 of this book.

Comparisons of Cotton Grown in Different Regions

Early studies (not mentioned above) led to a series of major studies aimed at defining the importance of the area of growth in determining the quality of cotton and its microbial flora. The later studies are discussed in the text that follows.

Microbiological Studies

Preliminary studies compared washed and unwashed cottons from three different growing regions and showed that unwashed cottons have high levels of GNB and endotoxin (Millner et al. 1983). Although unwashed cottons have higher levels of GNB and endotoxin, it is possible to select (using grade classification) unwashed cottons with high or low levels of GNB and endotoxin from specific

Table 44. GNB in cotton from different growing areas

State	Cultivar	GNB (log cfu/g)			
		First study		Second study	
		1981	1982	1983	1984
MS	Unspecified	6.65 (H)*			
	Unspecified	5.46 (L)			
	Deltapine 61		6.27	7.01	6.63
	Acala SJ5		6.74	6.13	6.42
	Growers Seed Assoc. 71		6.71	7.11	6.79
CA	Unspecified	4.33 (H)			
	Unspecified	3.47 (L)			
	Deltapine 61		5.16	5.17	6.17
	Acala SJ5		5.39	5.24	6.12
	Growers Seed Assoc. 71		5.34	5.19	5.90
	Acala SJ1		---	5.30	---
TX	Unspecified	6.29 (H)			
	Unspecified	5.81 (L)			
	Deltapine 61		5.75	6.98	5.60
	Acala SJ5		5.73	7.04	6.15
	Growers Seed Assoc. 71		6.43	7.03	6.38

*In 1981, 2 to 3 bales per cultivar per state were run in triplicate. Cotton cultivars used in 1981 at the various locations were not specified; instead they were chosen for their high (H) or low (L) bacterial counts and endotoxin levels.

regions of growth. In our studies two bales of cotton with high (grade 43) and low (grade 41) amounts of GNB and endotoxin were selected from the 1981 crop from Mississippi, Texas, and California. Again, higher counts were found in the cottons from Mississippi (table 44). The GNB counts in the cottons from Texas were also high.

The results from these early studies inspired the development of a major study in which three cultivars (Acala SJ5, Deltapine 61, and Growers Seed Association 71) were grown side by side in the California San Joaquin Valley, Mississippi Delta, and Texas High Plains in 1982 (Fischer and Jacobs 1985). For all three varieties the counts of GNB on lint and bracts were higher in Mississippi than in the other two sites. Also, the bracts had higher GNB counts than the corresponding lint. Levels of gram-negative bacilli and endotoxin are influenced by area of growth of the cotton regardless of the variety of the cotton grown (Millner et al. 1983). Low levels in the 1982 Texas cottons were probably the result of unusual weather conditions, since previous studies of cottons from this area had shown higher levels of GNB (Simpson and Marsh 1984).

This study was repeated in 1983 and 1984. Comparison with the data from 1981 and 1982 is shown in table 44. All counts are listed as logarithms. [The GNB data for 1981 and 1982 in table 44 were reported previously in colony-forming units per gram of cotton (cfu/g) rather than in log cfu/g.]

Table 45 includes the standard deviations for the counts from the 1982, 1983, and 1984 crop years. There is considerable overlap, but the 1983 counts for Deltapine 61 from Mississippi and for all varieties from Texas are significantly higher. Growers Seed Association 71 produced the highest levels of GNB in Mississippi for all 3 yr combined. In Texas levels of GNB for all three varieties of cotton were highest in 1983. Cottons from California had markedly lower levels of GNB regardless of the variety studied in 1982 and 1983. The 1984 levels of GNB on cottons from California were somewhat higher and approached those on cottons from Texas in 1984.

Bracts from these three cottons—Deltapine 61, Acala SJ5, and Growers Seed Association—were also studied (data not shown). Bracts from Mississippi cottons had GNB levels of 10^8 cfu/g in 1982 and 10^7 cfu/g in the other 2 yr, and bracts from California cottons had GNB levels of 10^3 to10^4 cfu/g in 1984 and 10^5 cfu/mg in 1982. Bracts from Texas cottons had GNB levels of 10^6 cfu/g except in 1983 when they contained 10^7 cfu/g.

Other cultivars of cotton were also studied in 1982. These cottons were harvested from Mississippi and California. The GNB levels for these cultivars are listed in table 46. Although the cultivars (excluding McNair) are not the same, the California cottons in 1982 contained two logs less GNB than the Mississippi cultivars. This is consistent with the differences seen in the larger studies.

In 1986 other cottons were sampled in an attempt to identify an area with low levels of GNB. The sampled cottons were grown in Mississippi and Arizona. The GNB levels from these cottons are shown in table 47. The GNB counts from cotton harvested in Arizona were not different from those from cotton harvested in Mississippi. GNB counts in 1986 in both locations were not significantly different from GNB counts in 1982 in Mississippi.

The specimens from the 1981 and 1982 crops were also studied by Millner et al. (1983, 1984, 1985). They used a most-probable-number technique, followed by replica plating on gram-negative-selective agar. Their data are similar to the data presented in table 47 and are shown in table 48. Millner and colleagues also tried to relate the airborne levels of GNB in human exposure chambers to the counts of GNB in the lint and concluded that GNB levels in lint generally could not be used to predict GNB levels in airborne dust generated from cards (Perkins et al. 1986).

Table 45. GNB in cotton from different growing areas

State	Variety	GNB (log cfu/g) (standard deviations in parentheses)		
		1982	1983	1984
MS	Deltapine 61	6.27 (0.25)	7.01 (0.23)	6.63 (0.26)
	Acala SJ5	6.74 (0.21)	6.13 (0.82)	6.42 (0.31)
	Growers Seed Assoc. 71	6.71 (0.20)	7.11 (0.25)	6.79 (0.29)
CA	Deltapine 61	5.16 (0.18)	5.17 (0.16)	6.17 (0.10)
	Acala SJ5	5.39 (0.30)	5.28 (0.33)	6.12 (0.30)
	Growers Seed Assoc. 71	5.34 (0.25)	5.19 (0.42)	5.90 (0.27)
	Acala SJC 1982	4.74 (0.21)		
	Acala SJ1 1983		5.30 (0.37)	
TX	Deltapine 61	5.75 (0.40)	6.98 (0.18)	5.60 (0.44)
	Acala SJ5	5.73 (0.21)	7.04 (0.18)	6.15 (0.13)
	Growers Seed Assoc. 71	6.43 (0.27)	7.03 (0.22)	6.38 (0.37)

Table 46. GNB in cotton cultivars grown in Mississippi and California in 1982

Mississippi		California	
GNB (log cfu/g)	Cultivar	GNB (log cfu/g)	Cultivar
6.55	Delta Experiment St. 56	4.47	Acala SJ–2*
6.18	Delta Experiment St. 188	3.99	Acala SJ–2*
6.56	Delta Experiment St. 422	4.37	Acala SJ–5
6.48	Stoneville 506	4.64	Stoneville 213*
6.57	Stoneville 825	4.13	Stoneville 213*
6.55	Deltapine 41	4.37	Lockett 77
6.62	Deltapine 55		
6.52	McNair	4.30	McNair

*Cultivars in this list that are identical (that is, they are listed twice) were grown in different locations in California.

Table 47. GNB in cotton cultivars grown in Mississippi and Arizona

GNB (log cfu/g) (standard deviations in parentheses)		
Mississippi 1982*	Mississippi 1986	Arizona 1986
6.55	6.42 (0.28)	5.82 (0.09)
6.18	5.82 (0.07)	5.95 (0.19)
6.56	6.53 (0.20)	5.84 (0.09)
6.48	5.67 (0.19)	6.10 (0.21)
6.57	6.25 (0.15)	6.24 (0.57)
6.55	7.09 (0.07)	6.05 (0.06)
6.62	6.58 (0.12)	6.24 (0.08)
6.52	6.38 (0.11)	6.08 (0.14)
	6.16 (0.05)	
	6.39 (0.20)	
Mean 6.50	6.33	6.04

*This column of data included for comparison.

The cottons used in these studies were also used in a number of other studies. These studies are summarized in the text that follows.

Pulmonary Function Studies

Pulmonary function studies were performed on healthy human volunteers in the model cardroom at Clemson, SC, to observe the effects of dust. These studies were sponsored by the National Institute of Occupational Safety and Health and were performed by various investigators. The studies evaluated the effects of dust from high- and low-grade cottons (grades 43 and 41) grown in 1981 in Mississippi, Texas, and California. The preliminary con-

Table 48. Levels of GNB in cotton fiber and airborne cotton dust samples

State-grade	GNB in 1981 samples of: Cotton (log MPN*/g)	GNB in 1981 samples of: Dust (log cfu/m³)	Variety	GNB in 1982 samples of: Cotton (log MPN*/g)	GNB in 1982 samples of: Dust (log cfu/m³)
MS–43	6.36	4.36	Deltapine 61	6.06	4.35
–41	4.55	3.01	Acala SJ5	6.72	4.44
			Growers Seed Assoc. 71	6.79	4.28
CA–43	5.40	3.05	Acala SJ5	4.65	3.55
–41	3.62	2.67	Acala SJ5	4.65	3.55
–21 (H)†	3.62	2.60	Growers Seed Assoc. 71	5.76	3.82
–21 (L)†	2.98	2.55	Acala SJ1	4.32	3.28
TX–43	6.85	3.77	Deltapine 61	5.71	3.58
–41	6.27	3.81	Acala SJ5	6.47	3.63
			Growers Seed Assoc. 71	5.51	3.93

*MPN, Most probable number technique, described in Millner et al. (1983).
†H, High bacterial counts and endotoxin levels. L, Low bacterial counts and endotoxin levels.

Source: Millner et al. (1983, 1984, 1985).

clusion was that significant differences in pulmonary function were due to grade and not to area of growth. However, the effect of grade was not significant for the California cottons (Bragg et al. 1983).

Differences in endotoxin content occurred based on the geographic location of the cotton (Olenchock et al. 1983). Cottons from Texas produced airborne cotton dusts with 390 ng endotoxin per mg of dust. The level of endotoxin was 168 ng/mg of dust from Mississippi cottons and only 40 ng/mg of dust from California cottons. Studies on endotoxins in airborne dusts liberated from these cottons showed marked differences among the grade-43 carded cottons from the three different growing regions, and these differences were reflected in changes of pulmonary function of healthy volunteers (Castellan et al. 1984). Airborne concentrations of bacteria (GNB and total), vertically elutriated gravimetric dust, and vertically elutriated endotoxin showed a statistically significant relationship to exposure-related acute declines in pulmonary function. The correlation coefficient for endotoxin was –0.94 and for GNB (in cfu/m³) was –0.91; gravimetric dust was the least correlated (coefficient of –0.34) (Castellan et al. 1984). Fungal content of air showed no correlation.

A subsequent paper (Castellan et al. 1988) confirmed that lower levels of airborne endotoxin are liberated in mills processing western cottons than in those processing Mississippi cottons.

Acute pulmonary function changes in the model cardroom at Clemson correlated better with concentrations of elutriated endotoxins than with gravimetric dust levels (Simpson and Marsh 1984).

In 1983, the same three varieties of cotton were studied again in the same three areas. The geographic area of growth of the cotton had a marked effect on the endotoxin contamination of the carded dust. The highest concentrations of endotoxins were in the dusts from carded Acala SJ5 cotton regardless of area of growth (Millner et al. 1984). Also levels of endotoxin from all three varieties of cotton from Texas were higher in 1983 than in 1982. No bacterial counts were given. The dusts collected from the card were studied in a model cardroom by Perkins et al. (1986), and they concluded that the relation-

ships between levels of viable bacteria and endotoxin in cotton lint and the levels in card-generated dusts were not definitive.

In 1984, the three varieties of cotton were again planted in the three locations described above. No human volunteer studies were done on the carded dust from this year.

Chemical Studies

The cardroom dusts from the cottons harvested from the 1983 field studies were also used for chemical studies (Domelsmith et al. 1985). California cardroom dusts had high levels of cellulose compared to the dusts from the Texas and Mississippi cottons. Variations in ash content correlated with variations in silicon, iron, and aluminium contents, indicating that soil contamination occurred. The profiles on gas chromatography differentiated the cottons by area of growth. Samples from California had relatively high levels of malate and citrate and low levels of mannitol. Samples from Mississippi and Texas had low levels of malate and high levels of mannitol.

Dust from bracts from the 1982 and 1983 studies were also evaluated. Differences were found in the levels of mannitol and malic acid in dusts from different areas (Domelsmith and Wood 1986). No consistent difference in terpenoid aldehyde concentrations occurred among locations or crop years (Bell et al. 1986). Concentrations of lacinilene, lacinilene methyl ether, and their precursors in bracts from Mississippi were higher than those in bracts from Texas, which in turn were higher than those in bracts from California (Greenblatt and Bell 1986).

Field Study

Differences in bacteriological and chemical findings inspired a major study to evaluate the cotton soon after it was sampled in the field, to sample throughout the fruiting period of the cotton plant, and to measure weather variables that might play a role in the differences found. The cotton in this study was grown in the same three areas plus College Station, TX. The cultivars were appropriate to the individual growing areas but were different from those grown in other test areas. The results of the study are reported in chapter 11 (figs. 1 to 4 and 6 to 9 in chapter 11 summarize the data relevant to this discussion).

Summary

The studies discussed in this chapter required considerable effort by many investigators to evaluate the effects of using different cotton cultivars and planting in different areas and years. Area of growth was an important variable as was crop year (as demonstrated in the data from Texas). The human pulmonary function data correlated the decreases in FEV_1 with the endotoxin content of the air of the exposure chamber. The chemical studies indicate the importance of soil contamination as a source of microorganisms and specific elements. The mannitol and malate (malic acid) differences may have been due to the multiplication of bacteria and fungi on cotton cultivars as harvest approaches.

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Chapter 11. Summary of Studies Evaluating Bacterial Colonization and Endotoxin Levels on Cotton Fiber

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Since field-grown cotton is generally contaminated with bacteria and other microorganisms (Morey et al. 1983, Simpson and Marsh 1988), it follows that attempts to minimize worker exposure to agents eliciting byssinotic symptoms might include strategies to reduce the microbial contamination of cotton fiber. A previous study suggests that high levels of bacteria on fiber occur late in the growing season and are increased by events such as a freeze, a frost, or application of a desiccant/defoliant prior to harvest (Morey et al. 1983). If microbial colonization of the fiber occurs as a result of some manageable factor in the field, it might be possible to intervene in the colonization process or to harvest prior to the outbreak of heavy contamination after boll maturation and fiber emergence.

A multilocation and multiyear investigation was undertaken to determine the seasonal dynamics of bacterial colonization of cotton fiber in the field. Gram-negative bacteria (GNB) and endotoxin were evaluated on samples of cotton fiber collected on a weekly basis throughout the growing season for 3 consecutive yr at College Station and Lubbock, TX; Mississippi State, MS; and Fresno, CA. Published papers and presentations generated from these studies are listed in "Other Sources" at the end of this chapter (unless they are already listed in the reference section and cited in this chapter).

Materials and Methods

Soil and Agronomic Practices

The cotton in this study was grown using conventional agronomic practices for the crop and the region. Cultivars used included Stoneville 112 (in 1986) and Stoneville 825 (in 1987–88) in College Station; Paymaster 404 in Lubbock (in 1986–1988); DPL 41 in Mississippi State (in 1986–1988); and Acala SJ–2 in Fresno (in 1986–1988). In all 3 yr of the study, unopened bolls (just before the period when the first bolls began to "crack" or dehisce) on the lowermost fruiting branches were tagged for later collection throughout the season. Only the first two bolls on the second and third fruiting branches (boll positions 2.1, 2.2, 3.1, and 3.2) and the first boll on the fourth fruiting branch (boll position 4.1) were tagged. These bolls normally open within 3 to 12 days after the first boll (boll position 1.1) opens. On each collection date (at least once per week), 5 individual tagged bolls from each of 16 sectors comprising the field plot were harvested (80 bolls total for each collection). In 1986 all bolls were ginned by hand in the laboratory using aseptic procedures. In 1987 and 1988, a small mechanical gin was used to separate the seed from the lint. The gin was thoroughly cleaned between samples using compressed air and ethyl alcohol. The lint was subdivided into three approximately equal portions for bacteriologic analysis, endotoxin analysis, and dry weight determination.

In 1987 only, closed bolls were collected from the lower fruiting branches (the "horizontal" samples), and all open bolls were collected from the plant (the "vertical" samples) for analysis. This modification of the experimental design was done because in the first year of the study rapid colonization of the fiber took place after boll opening—something that had not occurred in published reports (Fischer et al. 1980). It was hypothesized that the horizontal samples were rapidly colonized because they consisted of the bolls that were in closest proximity to the soil and to standing water after heavy rains. As the harvest season progressed, the number and proportion of vertical samples rapidly increased as more bolls opened. The vertical samples represent lint that is more characteristic of the entire plant and more representative of that which would be sent to the gin. Because the timing of boll opening was different for the vertical and horizontal samples, the length of exposure for potential contamination varied.

Each type of sample was collected weekly and evaluated for bacterial counts and endotoxin levels. The data recorded for horizontal and vertical samples were similar, and it seems doubtful that any statistical separations of the data from the two samples are likely. Therefore, only the horizontal samples are considered in this chapter.

Bacteriological Analysis

In GNB determinations, 2.5 g of lint were placed in 100 ml of phosphate buffer [pH 7.2; Fluorescence Treparenol Antibody (FTA) buffer, Baltimore Biological Laboratories] containing Tween 80 (0.1 percent w/v) [Tween 80-Fluorescence Treparenol Antibody (T–FTA)] and were shaken for 20 min on a rotary shaker at room temperature. Serial dilutions were prepared and plated on tryptic soy agar containing cycloheximide (400 μg ml^{-1}) plus vancomycin (15 μg ml^{-1}). Aliquots (0.10 ml) were spread on the agar surface, and the plates were incubated at 28 to 32 °C for 48 to 72 hr prior to counting the colonies. Bacterial counts were normalized by lint dry weight and expressed as $\log_{10}$ colony-forming units (cfu) per gram of dry lint.

Endotoxin Analysis

For endotoxin analysis, 2.5 g of lint was suspended in 50 ml of T–FTA made up using pyrogen-free water (PFW). A sterile pipette was used to physically submerge the lint in the buffer to enhance wetting for subsequent mixing. Samples were extracted by placing the extraction flask in a sonic bath for 10 min. Ten-fold and then two-fold serial dilutions of the extracted samples were prepared in T–FTA and analyzed by the Limulus amebocyte lysate (LAL) assay. Vigorous vortexing was done between each step and for each dilution. The same diluent was used for the endotoxin (lipopolysaccharide) standard. Samples were evaluated using a microtiter plate modification of the LAL assay (Kreeftenberg et al. 1977). Twenty microliters of sample extract (or dilution) were added to 20 μL of Limulus amebocyte lysate in a microtiter plate well, and the mixture was incubated at 37 °C for 60 min. After the mixture was incubated, 10 μL of 0.2 percent toluidine blue was added, and gelation was assessed by tipping the plates after 1 to 2 min. The ability to cause gelation (endotoxin-like activity) was visually compared to the ability of a standard (of *E. coli* endotoxin) to cause gelation. (The standard was obtained from Associates of Cape Cod, Woods Hole, MA.)

Results and Discussion

Bacterial Populations

The data from the 3-yr study of gram-negative bacterial populations associated with cotton fiber are summarized for each location in figures 1–4. Mean values for log cfu/g on lint from bolls harvested at least 1 wk after cracking (dehiscence) are shown in table 49. The following trends were observed for each of the 3 yr in all locations except Fresno. Following boll opening there was usually a rapid increase (within 7 to 10 days) in the number of GNB on cotton fiber. The increase was of several orders of magnitude, and after the period of increase the number of GNB remained relatively constant throughout the rest of the growing season. For these three locations, GNB generally accounted for 60 to

Figure 1. Number of GNB on cotton fiber after boll crack at College Station (CS), TX, in 1986–1988

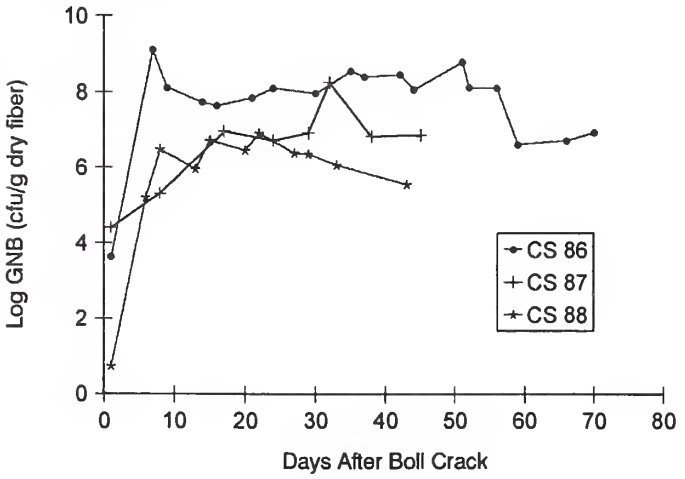


Figure 2. Number of GNB on cotton fiber after boll crack at Lubbock (Lbk), TX, in 1986–1988

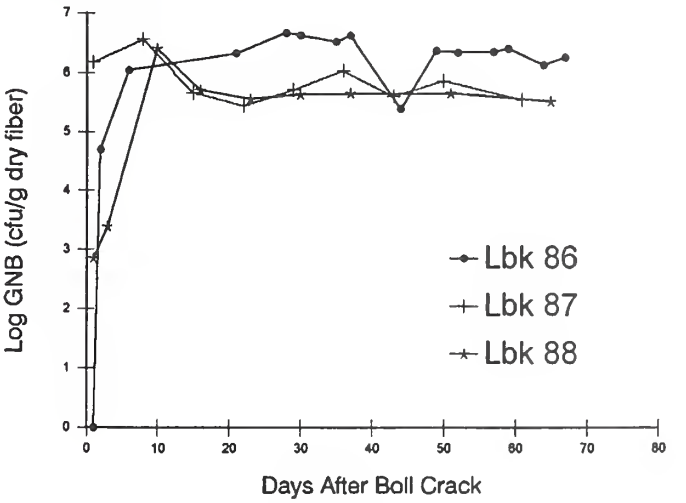


Figure 3. Number of GNB on cotton fiber after boll crack at Mississippi State (MS) in 1986–1988

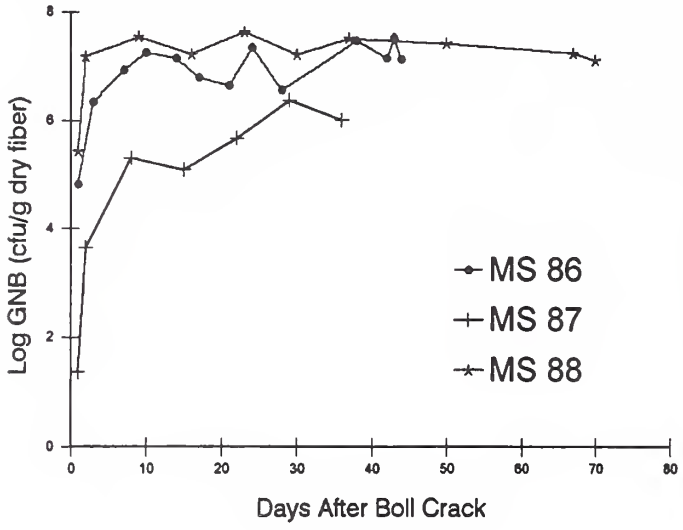
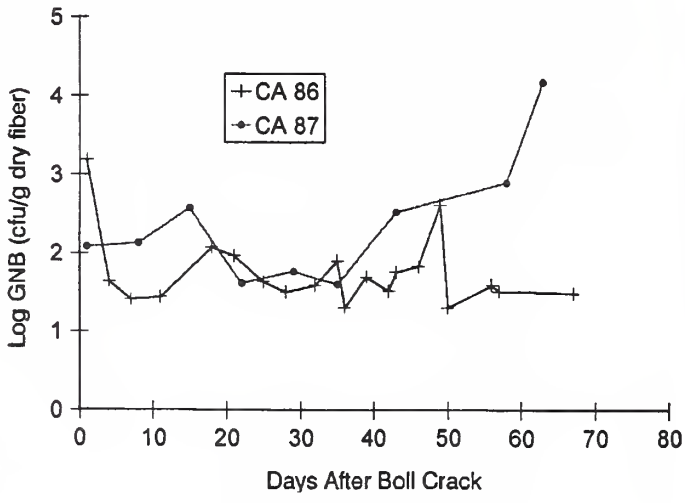


Figure 4. Number of GNB on cotton fiber after boll crack at Fresno, CA, in 1986–1987



90 percent of the total bacteria measured. However, the relative proportion of GNB to total bacteria measured in these studies may be related to the selectivity of the bacteriologic media used at the four locations. Although the general pattern of development of the bacterial population was nearly the same in all years, differences among years were observed and attributed to climatic events. For example, in 1987 at College Station, bacterial numbers rose more gradually than in 1986 or 1988, reaching a maximum in 20 days (fig. 1). This observation was attributed to a lack of significant rainfall during the early growing period (Zuberer and Kenerley 1993). Zuberer and Kenerley (1993) also speculated that higher bacterial counts observed at College Station in 1986 may be related to the more uniform distribution of rainfall throughout the growing season and the more dense and taller canopy that occurred in the 1986 crop.

In addition to the differences observed between years, effects of specific climatic events on bacterial numbers within a growing season were identified.

Table 49. Levels of GNB and endotoxin on cotton fiber

Location	1986	1987	1988
	Log GNB (cfu/g dry fiber)*		
Fresno, CA	1.7±0.3	2.4±0.8	3.2±1.2
College Station, TX	8.0±0.7	6.8±0.8	6.3±0.4
Mississippi State, MS	7.1±0.3	5.7±0.5	7.4±0.2
Lubbock, TX	6.3±0.3	5.8±0.3	5.7±0.3
	Log Endotoxin (ng/g dry fiber)*		
Fresno, CA	1.4±0.2	1.8±0.2	1.6±0.4
College Station, TX	3.7±0.5	3.5±0.6	3.6±0.2
Mississippi State, MS	3.2±0.4	3.2±0.3	4.2±0.2
Lubbock, TX	4.0±0.2	2.9±0.4	2.8±0.2

*Means and standard errors of means for fiber harvested at least 7 days after bolls cracked.

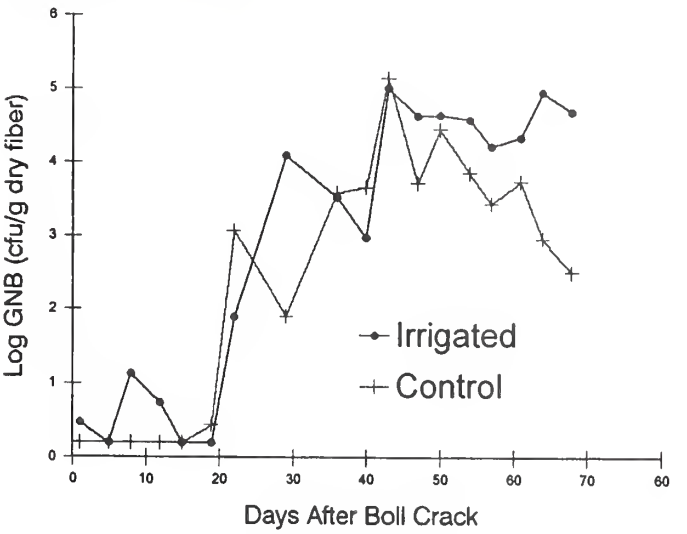
For example, at College Station in the 1987 growing year, bacterial numbers were observed to increase following intense rainfall events (for example, 32 days after boll crack, fig. 1). Similar patterns were observed for the other locations in Texas and Mississippi. Except for the responses to fiber wetting by rainfall, the impact of specific climatic events on the overall seasonal pattern of bacterial colonization appears to be minimal. These data suggest that annual seasonal growing conditions and climatic conditions are more important variables for determining the overall levels of bacteria on fiber than specific events.

At Mississippi in 1986 and 1987, populations of GNB on cotton fiber increased (0.7 log unit) after a defoliant was applied in the field (Borbon Reyes et al. 1988). At Lubbock in 1986, gram-negative bacterial numbers on cotton bracts dropped more than two orders of magnitude following a hard frost. The frost had little effect on gram-negative bacterial numbers on cotton fiber (Heintz et al. 1988).

The influence of seasonal climatic events on bacterial numbers was also demonstrated in two unique experiments designed to evaluate the effect of rainfall on bacterial populations on lint. Heintz et al. (1990) studied cottons protected from rainfall and overnight dew formation by a mobile rainout shelter. They observed that bacterial levels in the sheltered cotton were several orders of magnitude lower than those in cotton that was not sheltered. In the 1988 trial and a follow-up field trial in 1989 at Fresno, Harding observed that bacterial counts were higher on the lint from cotton plants in fields that were sprinkled twice a day for 30 min (a total of 0.6 cm water/day) than on lint from adjacent unsprinkled plots (Adelman and Harding 1989). Bacterial counts were significantly correlated with fiber moisture content (r^2=0.25; p<0.01). These results were obtained even though overhead sprinkling did not result in a statistically significant increase in fiber moisture content at sampling time.

At Fresno, the bacterial levels on fiber were at least two orders of magnitude lower than on fiber grown at the other locations (table 49 and figs. 4 and 5). The pattern of colonization also differed from the pattern observed at the other locations. In 1986 the highest bacterial levels were observed on the cracked boll sample, after which there was a general

Figure 5. The effect of overhead sprinkling on the number of GNB on cotton fiber after boll crack at Fresno, CA, in 1988



decline to a steady-state level for the remainder of the growing season. A similar pattern was seen in 1987 except the levels remained constant throughout the growing season from boll cracking through harvest. In response to speculation that the high temperature, low humidity, and lack of rainfall accounted for the lower bacterial levels on the fiber grown in the San Joaquin Valley, the 1988 experiment was modified to include plots treated with overhead sprinkling. As the bolls opened, gram-negative counts increased in both control and sprinkled plots. In the sprinkled plots, counts reached a plateau of approximately 4 x 10^4 cfu/g for the last 3 wk of sampling (fig. 5). In control plots, counts reached 10^4 cfu/g and declined the week before harvest. Therefore, increasing fiber moisture content (from overhead sprinkling) increased the gram-negative bacterial population size relative to the population levels in the control plot; however, the numbers for both plots remained below those observed at the other locations.

We further speculate that the increases in bacterial numbers on sprinkled plots were lower than those occurring in other locations due to the high temperature and low humidity that resulted in rapid drying of the sprinkled fiber. Overhead sprinkling also appeared to alter the composition of the gram-negative bacterial population relative to the population in control plots. Many different groups of

bacteria were identified in the analysis; however, most of the groups contained only a few isolates, and it is difficult to be confident about the observed population shifts.

The influence of climatic events on the fiber bacterial populations was also evaluated in a third study in which newly opened bolls were inoculated in situ with a rifampicin-resistant strain of *Enterobacter agglomerans* and exposed to or sheltered from rainfall (Batson et al. 1991). *E. agglomerans* is commonly found in cotton fiber throughout the world (Fischer and Kylberg 1983, DeLucca et al. 1988). Bacterial counts were evaluated on samples collected on a weekly basis for 4 wk after inoculation. As expected, bacterial counts were higher on exposed lint; however, there was a decrease in the rifampicin-resistant *E. agglomerans* compared with the total population over the sampling period. The bacterial counts on the sheltered bolls were lower, but the rifampicin-resistant *E. agglomerans* remained the dominant component of the population.

Endotoxin Levels

Figures 6–9 show the endotoxin determinations by sample day at each location for each of the 3 yr (1986, 1987, 1988) (Fresno, 1986 and 1987 only). As was true for the bacterial populations, there were distinct patterns of accumulation of endotoxin on the lint. In general, for all locations except Fresno, there was an initial increase in endotoxin levels (ng/g) within 1 to 2 wk after boll opening, followed by a steady state level of endotoxin on the fiber throughout the remainder of the growing season. Mean values for endotoxin content on lint from bolls harvested at least 1 wk after cracking are shown in table 49.

For the Fresno samples, endotoxin levels on the samples harvested early were not different from those of samples collected later in the growing season (figs. 9 and 10). Endotoxin levels were comparable for irrigated and nonirrigated (control)

Figure 6. Endotoxin content of cotton fiber after boll crack at College Station (CS), TX, in 1986–1988

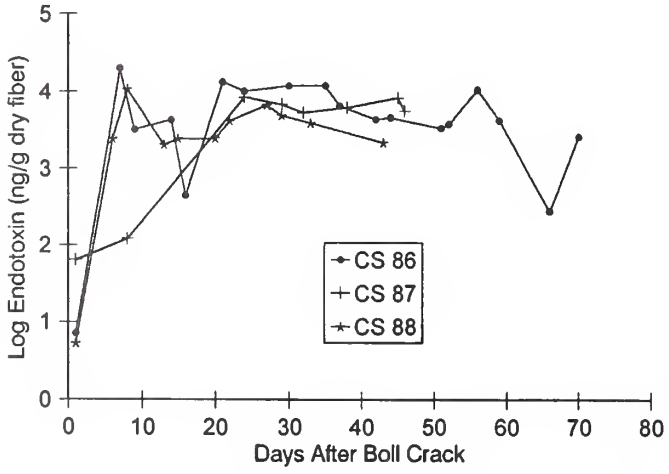


Figure 7. Endotoxin content of cotton fiber after boll crack at Lubbock (Lbk), TX, in 1986–1988

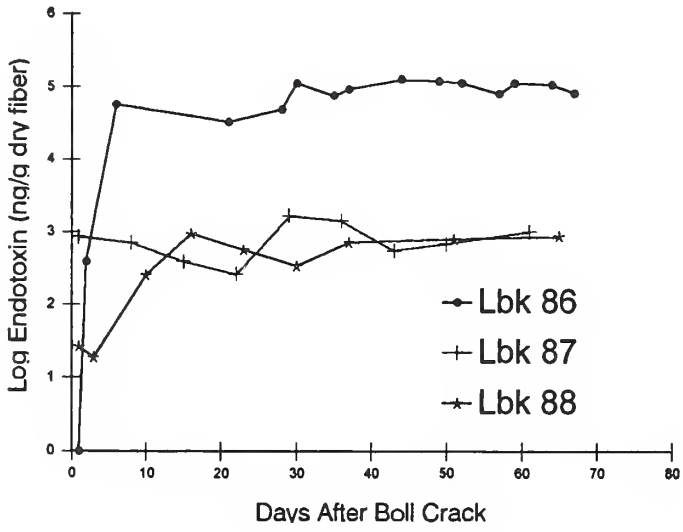


Figure 8. Endotoxin content of cotton fiber after boll crack at Mississippi State (MS) in 1986–1988

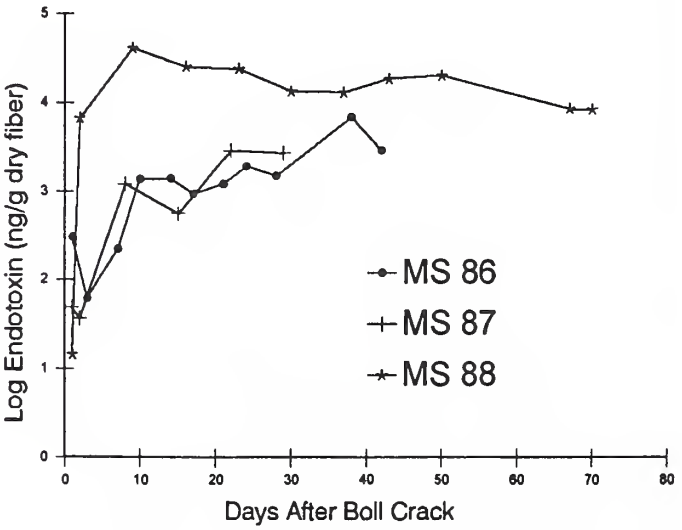


Figure 9. Endotoxin content of cotton fiber after boll crack at Fresno, CA, in 1986–1987

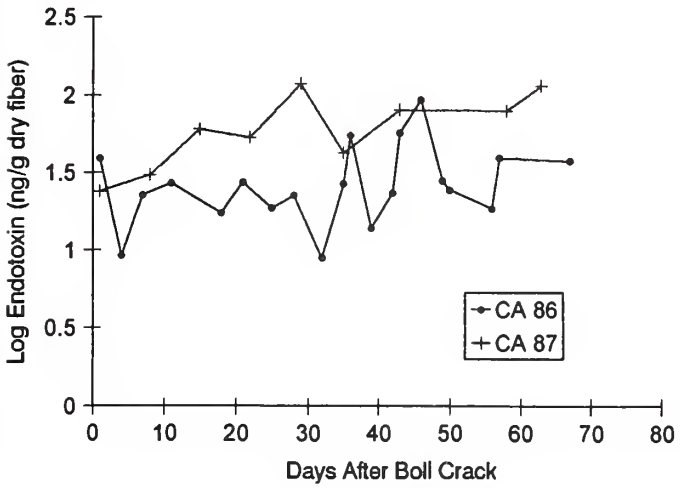
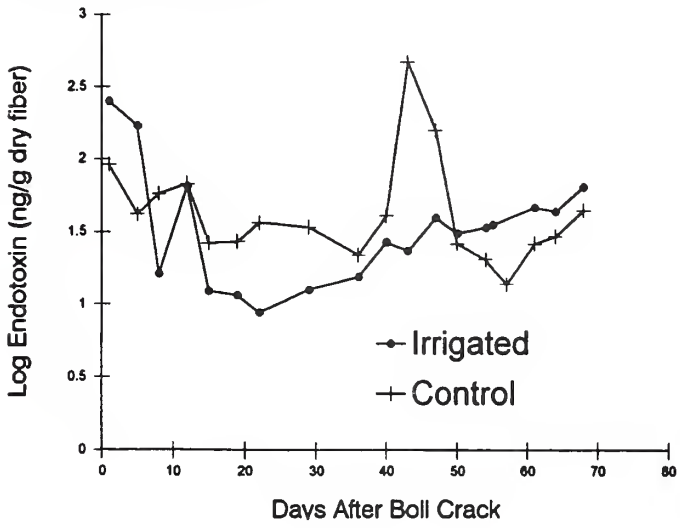


Figure 10. The effect of overhead sprinkling on endotoxin content of cotton fiber after boll crack at Fresno, CA, in 1988



samples (fig. 10). Within a sampling day, the standard deviation of the mean levels were always larger for the California data than for the data from other locations. While this may reflect the variability of endotoxin within a field, it more likely reflects the experimental error associated with endotoxin measurements at the lower concentrations found at Fresno. The endotoxin levels for California were always one to two orders of magnitude lower than the levels for other locations.

Differences in endotoxin levels between sampling years were observed for each of the locations other than California, and, as discussed for the bacterial populations, these differences may be due to the influence of climatic events. Although there were some points at which increased bacterial numbers were not reflected in increased endotoxin contents, in general the endotoxin values followed the same trend as bacterial numbers. This was expected since endotoxin is a constituent of gram-negative bacterial cell walls, and therefore the curves for endotoxin levels should closely parallel the curves for bacterial levels. Thus, the use of bacterial counts, either total or gram-negative, appears to be a good indicator of the pattern of endotoxin accumulation on fiber. The points where endotoxin and bacterial cell counts do not track together are likely to be a result of random sample variation.

Defoliant application or frost did not alter the overall pattern of endotoxin accumulation on cotton fiber. A small rise in endotoxin levels (0.2 log units) was observed following defoliant application in Mississippi in 1986. However, no corresponding increase was observed in 1987 even though the rise in gram-negative bacterial numbers was comparable for both years. In 1986 a late-season, hard frost did not affect endotoxin levels on cotton fiber at Lubbock.

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Chapter 12. Bacteria in Lancashire Cotton Mills: 1950–1990

A.F. Tuxford

The first part of this chapter reviews the techniques and results of earlier studies that identify the microorganisms found on cotton and in dust and air. The second part reviews more recent work (some not published elsewhere) on gram-positive bacteria and includes an investigation into the cytotoxicity of *Bacillus* species present in cotton mills.

Critique of Previous Studies in Lancashire Mills

1950–1960

Furness and Maitland (1952) examined the microbial content of 13 cotton and mill dusts (mainly "fly," that is, larger particles of cotton fiber and bracts removed by cotton-cleaning machinery). They also evaluated finer dust material that passed through a 90-mesh sieve and that had been collected by air-cleaning plants in the mill. Only gram-positive bacilli and cocci were seen on the gram-stained films; bacterial and mold spores, probably *Aspergillus niger* and *Penicillium* species, were also observed. On nutrient agar, *Bacillus* species predominated with several varieties of gram-negative bacteria (GNB). Micrococci were isolated once and *Actinomyces* species on three occasions. Some *Bacillus* species grew on the MacConkey plates where the predominant genus was *Bacterium* (as defined by Wilson and Miles 1946), mainly atypical *Bacterium aerogenes* with a few *Bacterium alkaligenes* and *Achromobacter*. No pattern relative to the grade of cotton or collection site was discernible.

Among the 21 species of *Bacillus* identified were 2 *Bacillus megaterium*, 1 *B. pumilus,* and 18 that resembled *B. pumilus*, *B. subtilis,* or *B. coagulans* (Furness 1951). Five strains of *Clostridium*, including *Clostridium welchii* (*Cl. perfringens*) type A, were isolated. The Czapek Dox plates were often overgrown with *Mucor* species, but *Aspergillus* species, including *Aspergillus niger* and *Penicillium*, were also present. There were wide variations among the relative percentages of the fungal isolates in the different samples.[1]

Drummond and Hamlin studied airborne bacteria using a Bourdillon-type slit sampler loaded with 5 percent nutrient agar to prevent the colonies from spreading (Drummond and Hamlin 1952, Hamlin 1952). The samples were incubated aerobically at 37 °C. Microorganisms were assigned to genera using Bergey's classification (Bergey 1948).[2] In the cardrooms the bacterial counts were several thousand colony forming units per cubic foot (cfu/ft^3), reduced to 500–1,000 cfu/ft^3 where speed frames were in the same room. In spinning rooms and weaving sheds the counts were 50–200 cfu/ft^3 (such as might be found in canteens or crowded offices), but the species were different than those found in cardrooms. In a mill that processed only viscose rayon, counts of airborne bacteria were <10 cfu/ft^3 during carding, but in cotton mills carding both cotton and rayon the average count rose to 420 cfu/ft^3. In mills that reprocessed waste cotton, two sample counts on the breaker cards were 41 and 230 cfu/ft^3, and in precarding areas (devil rooms) they were 400 and 1,700 cfu/ft^3. Counts for four blow rooms ranged from 950 to 12,000 cfu/ft^3 and for a bale-opening room and mixing chamber were 79 cfu/ft^3 and 1,300 cfu/ft^3, respectively. Six mills spinning mixtures of American and Brazilian cotton were visited several times; the average count in the cardrooms was 11,700 cfu/ft^3. No obvious correlation existed between the source of the cotton and the microbial counts in the carding rooms. The bacterial numbers on American and Brazilian cottons varied between the 1949 and 1950 seasons.

One mill spinning cotton predominantly of American origin and another spinning Sudanese cotton were studied in greater detail (Drummond and Hamlin 1952). Table 50 shows the results from one visit to each mill or weaving shed with the original

[1] The second part of Furness' thesis contains the microbiology with insufficient detail to identify the species according to modern criteria. Bergey's (1948) classification was used to assign the organisms to genera; the genus *Bacterium* contained both gram-positive and gram-negative bacilli that did not produce spores.

[2] This edition lists some species of *Flavobacterium* and *Achromobacter* as gram-positive. Prindle (1935–36) also assigned some gram-positive species to *Flavobacterium*.

Table 50. Airborne bacterial counts in mills that process spinning, weaving, and waste cotton

Bacteria	Bacterial counts (thousands of cfu/m³ air)					
	Waste cotton Carding room	Sudan cotton Carding room	U.S./Egypt Cotton blend* Carding room†	U.S./Egypt Cotton blend* Ring room†	Unknown cotton Weaving shed	Egypt cotton Mix Weaving shed
Bacillus	7.8	81.2	137.7	4.9	7.5	3.1
Corynebacteria	<0.1	0.4	9.2	0.4	0.2	0.4
Micrococcus	5.7	1.5	3.9	0.2	0.5	0.7
Actinomyces	0.0	8.1	3.9	0.0	0.1	0.1
Achromobacter	0.1	0.0	3.9	0.2	0.1	0.4
Aerobacter	0.1	0.0	20.5	0.5	0.2	0.1
Flavobacterium	0.0	0.0	1.0	<0.1	<0.1	0.2
Nonviable	<0.1	4.6	3.9	1.5	0.0	0.1
All bacteria‡	81.0	102.4	218.9	7.8	8.7	5.2

*Blend consisted of 58 percent U.S. cotton and 28 percent Egypt cotton.
†The carding rooms and ring rooms from which the U.S./Egypt cotton was sampled were part of the same mill.
‡Total counts were the original viable counts and include other (presumably) unidentified microorganisms.

Source: Hamlin (1952).

counts per cubic foot converted to thousands of cfu/m^3 to allow for comparison with other researchers' results (35.31 ft^3 = 1 m^3). *Bacillus* species outnumbered GNB by a factor of 10 in the carding rooms and also predominated in the much lower counts in the ring rooms and weaving sheds. A small proportion of the cardroom cultures and nearly 20 percent of the ring room isolates were lost before identification. Neither anaerobic nor human respiratory bacteria were found.

Drummond et al. (1954) studied airborne bacterial counts before and after oiling of cotton was introduced to reduce airborne dust. After oiling was introduced, four of the six mills had significantly reduced levels of airborne bacteria (in one mill the count was higher). One year later, the bacterial counts after oiling in most of the mills were significantly higher than in the previous year. However, the increase was thought to be due to an alteration in the bacterial flora of that year's cotton crop.

1960–1970

Tuffnell (1960) studied two cotton mills where the incidence of byssinosis in the cardrooms was known; one processed coarse cotton, the other spun fine cotton. In the mill that processed coarse cotton, 72 of the 135 cardroom workers (47 percent) had byssinosis whereas only 5 of the 32 cardroom workers processing fine cotton (16 percent) had byssinosis. Two jute mills in Dundee, Scotland, with mean dust counts of 325 particles/ml of air and 182 particles/ml, served as controls. Six to eight plates containing 1 percent agar dissolved in tap water were exposed for 30 min during the mill shifts and then stored on ice. After homogenization in saline, the suspension was inoculated onto meat-extract peptone agar incubated for 24 hr at 37 °C, then for 24 hr at 22 °C to isolate bacteria. Dextrose peptone agar plates with Rose Bengal and streptomycin added were incubated at 22 °C for 3 days for isolation of fungi. Homogenization of the tap water agar increased the colony counts by a factor of 3.9 to 7.4 (mean 5.3). Throughout the day, air was drawn onto filter paper through a Hexhlet apparatus. The paper was later macerated and inoculated onto the culture media. The counts obtained tended to be lower possibly due to the clumping of microorganisms or desiccation during collection.

In table 51 Tuffnell's results have been converted to numbers of viable organisms per cubic meter and, for comparison with other researchers' results, have been reduced by a factor of 5.3 (the mean of the increases due to homogenization). This has produced some discrepancies compared with the original tables. Although homogenization raised the counts, the use of a transport medium with refrigeration may have reduced viability. *Bacillus pumilus* strains predominated in the cotton mills; *Bacillus megaterium* predominated in the jute mills. A definite association was found between the number of live organisms of *Bacillus pumilus* and *Bacillus subtilis* in the cardrooms and the incidence of byssinosis. There was no relationship between the total numbers of bacteria and fungi in the air of the cardrooms and byssinosis. No comment was made about the absence of GNB reported by other researchers.[3]

[3] Gram-stained films from the inoculation material would have been desirable. It is not known why Tuffnell combined *Bacillus pumilus* with *Bacillus subtilis*. Perhaps he accepted the view of Wilson and Miles (1955) that the two species were so closely related that it was not possible to separate them.

1970–1980

Lacey and Lacey (1987) compared a traditional Lancashire cotton mill (one visit) to another mill in Northeast England. The second mill consisted of a new single shed with partitioned areas that were later rearranged in a linear fashion so that scutching was separated from carding and speed was separated from ring frames. During the study the new mill went on short time (reduced work schedules), and the standard of cleanliness diminished. Eight visits between May 1975 and April 1978 were made to collect 32 cotton dust samples and 143 air samples for bacterial counts. Air samples were collected with a cascade impactor (used for spore counts) and an Andersen sampler (loaded with malt agar plus penicillin and streptomycin or with half-strength nutrient agar plus cycloheximide or with tryptone soya casein hydrolysate agar plus cycloheximide). Incubation was carried out at 25 °C for 7 days, 40 °C for 6 days, or 55 °C for 3 days. Colonies were counted and classified according to appearance and microscopic features, and selected bacterial colonies were identified using API 20E (avail-

Table 51. Viable bacterial and fungal counts in the air of cotton mills processing cotton and jute

	Viable counts (thousands of cfu/m³ air) in mills processing:			
Bacteria or fungi	Coarse cotton 12‡	Fine cotton 7‡	Jute* 7‡	Jute† 3‡
Bacteria				
B.pumilus /*B. subtilis*	184.5	20.0	12.7	1.3
B.megaterium	0.0	0.0	6.6	6.6
Micrococcus	43.6	1.1	30.4	5.4
Actinomy	14.6	0.0	105.6	3.2
Total bacteria§	242.4	22.0	215.8	20.6
Fungi				
Aspergillus	4.7	1.7	14.7	3.2
Penicillium	6	1.6	37.6	8.8
Total fungi§	15.3	3.4	58.7	13.3

*325 particles of dust/ml of air.
†182 particles of dust/ml of air.
‡Number of readings from which counts in this column were averaged.
§The total counts of bacteria and fungi comprised all species including those that were nonviable or not identified.

Source: Tuffnell (1960).

able from API BioMérieux La Balme Les Grottes, 38390 Montalieu Vercieu, France).

The predominant bacteria identified by Lacey and Lacey (1987) in the new mill were "gram negative yellow rods, mainly *Enterobacter agglomerans* and *Bacillus* species." Seven species of *Bacillus*, two species of *Pseudomonas*, two species of *Enterobacter*, two species of *Flavobacterium*, and three other species of GNB were identified. *Bacillus licheniformis* was present in nearly half of the cotton samples and one-fourth of the air samples. However, little evidence exists to show how these species' attributions were made, and the counts seem to have been performed on colored colonies irrespective of their gram reaction. Also there are a number of typographical errors that make interpretation difficult. The paper is more concerned with fungi. The predominant fungi were *Cladosporum* spp. in 63 percent of cotton dust samples and 76 percent of air samples, and all plates yielded greater than 3.3×10^3 cfu of *Cladosporum* spp. per gram of dust. *Penicillium* spp. were found in 72 percent of the cotton dust samples and in 74 percent of the air samples. *Aspergillus glaucus* group and *Aspergillus niger* were usually most numerous in the early stages of processing. Thermophilic actinomycetes were present in 78 percent of the 32 cotton dust samples.

In table 52, counts of cfu were calculated using the percentages in the fifth table of Lacey and Lacey. Unlike other workers they appeared to find the highest counts in the weaving sheds; however, because they did not identify the microbial species it is difficult to evaluate these results. The results for the two mills were not shown separately; hence this was a missed opportunity to show the development of the airborne microbial flora in a new mill.

A major study in Lancashire was done by Cinkotai et al. (1977) to evaluate air quality and the incidence of byssinosis in the workplace. Air samples from cardrooms and dusty workplaces of seven cotton spinning mills, two cotton waste mills, and five willowing mills were compared with air samples from a wool mill, a tea packing plant, and a pipe tobacco factory. Each workplace employed fewer than 100 workers. Airborne dust concentrations excluding fly were measured with a Rotheroe Mitchell sampler. A "Staplex" sampler (400 L/min) collected airborne endotoxin samples onto glass-fiber filters for assay by the Limulus lysate method by Rylander. Airborne bacteria and fungi were collected by an Andersen impactor (which contained nutrient agar having 1/3-strength Lab M plus 5 μg/ml nalidixic acid to select for gram-positive bacteria, Endo Agar plates with 0.5 units/ml penicillin to select for GNB,[4] and "5 percent malt agar" to select for fungi). Eight samples were collected at a height of 1 m from four sites within each workplace. After incubation for 1 day at room temperature, the nutrient and Endo Agar plates were then incubated for 1 day at 37 °C, and the malt agar plates were left at room temperature for another 2 to 6 days before the colonies were enumerated.

[4] Designed as a differential medium with lactose for the differentiation of the *Enterobacteriaceae*, Endo Agar inhibits gram-positive bacteria with fuchsin and sulphite. Susceptibility of both gram-positive and gram-negative species to different dyes varies.

Table 52. Estimated viable bacterial counts in air samples from two cotton mills

	Viable bacterial counts (thousands of cfu/m³ air) from colonies						
	Colony color						
Mill area	Yellow	Orange	Red	Buff	White	Other	Mean
Carding room	5.4	2.8	1.5	0.7	4.0	0.2	14.8
Ring room	7.0	3.9	1.4	0.5	2.4	0.2	15.1
Weaving shed	18.0	8.8	1.8	0.9	14.5	0.4	43.8

Source: Lacey and Lacey (1987).

Table 53. Viable counts of airborne bacteria and fungi, levels of dust and endotoxin, and prevalence of byssinosis in various types of mills

Mill type and number*	Viable counts (thousands of cfu/m³ air)			Dust (mg/m³ air)	Endotoxin (ng/m³ air)†	Prevalence of byssinosis (%)
	On nutrient agar	On Endo agar	Fungi			
Tobacco	2.10	0.49	0.95	0.35	0.00	0
Wool	15.00	0.18	2.50	1.89	0.00	0
Cotton 3	7.60	0.34	12.00	3.80	0.32	7‡
Cotton 7	9.70	1.60	4.40	2.47	0.20	12
Cotton 6	18.00	2.00	9.60	0.90	0.80	17
Cotton 5	16.00	2.90	3.80	0.64	0.80	21
Cotton 4	16.00	3.10	1.50	0.95	1.60	28
Cotton 1	26.00	2.80	7.10	0.46	0.62	33
Cotton 2	77.00	3.50	5.60	3.40	0.93	40

*Cotton mills were numbered for the purpose of distinguishing among them.
†Presumably ng/m³ air but text states that the units were mg/g.
‡Eighty-one percent questionnaire response compared with >85 percent in other mills and therefore of different statistical validity.

Source: Cinkotai et al. (1977).

Table 53 shows the results of Cinkotai et al. (1977), ranked according to the percentage of byssinosis in the workplace. There was no clear association between the prevalence of byssinotic symptoms and the concentration of airborne "fly-less" dust, nor between symptoms and the fungal count. Endotoxin levels in the cotton mills ranged from 0.20 to 1.60 "n/m³" in their table.[5] They found a good dose-response relationship between the prevalence of byssinotic symptoms and the number of colonies on the Endo Agar plus penicillin plates (r>0.95, p<0.001). They commented that if the high count of gram-positive bacteria in the wool mill were excluded, then the relationship between these microbes and the prevalence of byssinosis would be nearly as significant as that between GNB and the prevalence of symptoms. They concluded that the exact role of bacteria in the etiology of byssinosis was unclear and required further investigation.

The study of the Lancashire cotton mills was extended to include another 14 spinning mills (Cinkotai and Whitaker 1978). The methods were the same. Samples were collected for protease estimation by a spectrophotometric method. Viable counts on the two culture media were made on different days throughout the survey. Concentrations of airborne bacteria on Endo Agar (which predominantly selects for gram-negative species) averaged 270 to 6,150 organisms/m³ and on nutrient agar (which is believed to select for gram-positive species) averaged 1,200 to 89,300 organisms/m³. Bacterial numbers on both media correlated significantly with the prevalence of byssinotic symptoms

[5] Quoted as "0–1.6 mg/g" in the text of Cinkotai et al. (1977), these units are presumed to be in error as their table column is headed n/m³ (that is, nanograms per cubic meter). Other samples from outside the mills gave even higher endotoxin readings (Cinkotai and Franklin 1975). All sample containers, reagents, and glassware, etc., must be free of endotoxin when performing the Limulus lysate assay to avoid falsely high results.

($p<0.002$, $r>0.61$)[6] as did the protease content of airborne dust and particles of 2–4 μm diameter ($p<0.02$, $r>0.51$). Greater significances were observed when the number of years of worker exposures were related to the viable bacteria (Endo Agar $p<0.001$, $r>0.73$; nutrient agar $p<0.002$, $r>0.66$) or the protease values ($p<0.02$, $r>0.53$). Identification of the airborne bacterial species would have added to this useful paper, which shows the prevalence of byssinotic symptoms in the cardrooms during the 1970's and the decline in the bacterial populations compared with the 1950's.

Nicholson (1980) collected airborne microorganisms at 10 a.m. and 3:30 p.m. on 10 consecutive Mondays from 2 sites by the card machines and 2 sites by the draw frames. This project was supervised by Cinkotai and used the same methods previously described by Cinkotai et al. (1977). The previously unpublished viable counts are given in table 54 and show that the weekly variations on Endo Agar (presumed to be gram-negative species) are relatively small. Although the bacteriology was limited to counts on Endo Agar without species differentiation, it is important because it is the only study to show the weekly variations in bacterial numbers at the same sites over a long period.

[6] The statistically significant relationship between bacterial counts and byssinosis prevalence contradicts information in Tuffnell (1960) and requires further investigation. Note: Figures 1 and 2 in Cinkotai and Whitaker (1978) are transposed.

1980–1990

Two cotton spinning mills and a weaving shed and offices of another mill were studied by Cinkotai et al. (1984). Workplace dust levels were measured with Rotheroe Mitchell L60 samplers. Samples (28.3 L/min) for enumeration of airborne bacteria were collected on an Andersen six-stage sampler (Model 2,000; 28.3 L/min) loaded with nutrient agar. Plates were exposed for 1 min, incubated for 24 hr at 37 °C, and then left at room temperature for 24 hr before enumeration. The concentrations of bacteria in the general environment ranged from 1,300 to 150,000 cfu/m^3 (table 55). The concentration of airborne bacteria did not correlate with the concentration of airborne dust in the rooms.

Table 54. Airborne bacteria (on Endo agar) monitored for 10 consecutive Mondays in a mill that processed coarse cotton

	Airborne bacteria (thousands of cfu/m^3 air)			
	Carding area		Draw frame area	
Week	10 a.m.	3:30 p.m.	10 a.m.	3:30 p.m.
1	39.0	28.0	24.0	18.0
2	41.0	29.0	30.0	23.0
3	35.5	34.0	17.0	15.0
4	28.0	23.0	18.5	11.0
5	53.5	55.0	19.0	33.0
6	66.0	41.5	34.0	27.5
7	40.0	48.0	21.0	23.0
8	42.0	57.0	19.5	31.0
9	64.5	42.5	32.0	27.0
10	68.0	56.0	41.0	35.5
Mean	47.7	41.4	25.6	24.4
Std. dev.	14.1	12.5	8.1	7.9

Source: Nicholson (1980).

Table 55. Comparison of average personal dust levels and average workplace airborne bacteria in three mills (averaged over Monday, Wednesday, and Thursday shifts)

Location (& cotton grade)	Area	No. of workers		Dust level (mg/m^3)	Number of bacterial samples	Airborne bacteria (thousands of cfu/m^3 air ± std. dev.)
		Total	With byssinosis			
Mill 1	Carding, blowing	10	3	4.6±3.2	18	150.0±112.0
(Very low)	ring spinning	12	2	1.6±1.1	12	45.0±11.0
Mill 2	Carding, blowing	14	2	1.7±1.5	30	18.1±7.7
(Medium)	ring spinning	17	0	0.6±0.2	30	1.5±0.7
Mill 3	Weaving shed	Ns*	Ns	0.9±0.5	24	1.3±0.2
(Weaving)		28	0	5.1±2.8	12	2.4±0.2

*Ns, not stated.

Source: Cinkotai et al. (1984).

Additional visits were made to 31 mills by Cinkotai et al. (1988). Rotheroe Mitchell dust samplers were used to collect dust throughout 8-hr shifts. Samples were sent to Sweden for endotoxin estimation. Bacteria were cultured on nutrient agar and Endo Agar that contained 0.5 units/ml penicillin loaded onto an Anderson six-stage sampler. [Endo agar was unavailable, so MacConkey's medium plus the same concentration of penicillin was supplied (M. Khan, personal communication 1989).] Within an 8-hr shift, six samples exposed for 30 sec or 2 min were collected at a height of 1.5 m (that is, the approximate worker breathing zone). Plates were incubated for 24 hr at 36 °C and at room temperature for another 24 hr. Airborne bacteria were virtually absent in 21 factories and were below significant levels (5,000 cfu/m^3 total count and 300 cfu/m^3 GNB) in another 8 factories. Eleven of the mills in which the bacterial counts were high were revisited, and three yielded concentrations of GNB consistently greater than 300 cfu/m^3. In two mills that processed coarse cotton, the counts of airborne bacteria were 1,150 cfu/m^3 and 449 cfu/m^3. In the mill that processed medium-grade cotton, the airborne gram-negative bacterial count was 707 cfu/m^3. Total counts for these three mills were 3.56×10^4 cfu/m^3, 1.65×10^4 cfu/m^3, and 1.57×10^4 cfu/m^3. The average concentrations of endotoxins ranged from 0.01 $\mu m/m^3$ to 0.58 $\mu m/m^3$. In 14 out of 22 cardrooms, dust levels were greater than 0.5 mg/m^3 and breached official hygiene standards.[7]

Importance of Gram-Positive Bacteria in Cotton Mills

Differential colony counts for areas throughout the mills were performed by Moogan (1986) on the plates previously enumerated by Cinkotai et al. (1988). (Nutrient agar was used for the differential counts to include more fastidious bacteria suppressed on selective media.) When received, the plates had been incubated at 37 °C for 48 hr and left at room temperature for up to 1 wk before being stored at 4 °C. Moogan identified representative colonies. Gram-negative strains were inoculated into API 20E; API 20B was used for the gram-positive strains using the manufacturers' protocols. Other characteristics, for example, sporulation and lecithinase production, were tested as appropriate. Attributions were made from the API database for GNB. Cowan and Steel's Manual (Cowan 1974) was used for identification of the other species.[8]

[7] By implication airborne bacteria and endotoxin levels were not statistically significantly associated.

[8] The API system was designed primarily for hospital bacteriology and the databases are expanded as new species are described. There was no suitable database for the genus *Bacillus*. Like Tuffnell (1960), Moogan did not distinguish between *Bacillus subtilis* and *Bacillus pumilus*. Confirmation of some *Bacillus* species' identities has proven difficult when using the subsequently published *Bacillus* Atlas; Parry et al. (1983) used the first 12 tests of API 20E with additional tests. Prolonged incubation API 20B will alter the results (Hoult, personal communication 1988).

Table 56. Bacteria in the air of the card room or ring room in mills that process fine, medium, or coarse cotton or synthetic fiber

	Airborne bacteria (hundreds of cfu/m³ air)							
	Card room				Ring room			
Bacteria	Fine	Medium	Coarse	Synthetic	Fine	Medium	Coarse	Synthetic
B. polymyxa	31.4	25.2	45.6	33.4	1.1	2.1	6.9	6.8
B. licheniformis	15.6	5.0	22.9	11.4	0.5	0.8	3.6	2.7
B. cereus	1.4	2.0	9.0	6.5	0.0	0.7	0.4	6.8
B. coagulans	5.7	7.7	22.0	9.0	0.6	0.8	2.6	2.7
B. mycoides	18.6	4.7	23.1	4.3	0.8	0.2	3.6	0.9
B. laterosporus	8.2	0.9	0.8	0.0	0.0	0.2	0.3	0.0
B. pumilus/ B.subtilis	7.7	6.8	32.6	9.0	0.1	0.8	4.3	1.2
Nocardia spp.	3.2	0.4	5.3	1.6	0.1	0.1	0.8	0.2
E. agglomerans	18.3	19.9	38.8	23.1	0.7	2.3	7.2	2.7
E. cloacae	3.8	3.6	16.3	6.3	0.2	0.2	1.5	2.2
P. cepacia	3.8	1.1	22.7	4.6	0.1	0.3	3.2	0.8

Source: Moogan (1986).

The results of the survey were complex. Average counts for the air in card and ring rooms in which different grades of cotton (countries of origin were unknown) are handled are given in table 56. *Bacillus polymyxa* predominated, and high numbers of other *Bacillus* species and *Enterobacter agglomerans* were also present. The numbers of all species were highest in the mill that processed coarse-grade cotton. From the mills that processed fine-grade cotton to the mills that processed coarse-grade cotton the greatest increase was observed in numbers of *Bacillus subtilis* and *B. pumilus* (770 to 3,260/m³). In the cardrooms of mills, counts of *Enterobacter agglomerans* were twice as high when the mill processed coarse cotton instead of fine cotton, and counts of *Pseudomonas cepacia* were six times higher in mills processing coarse cotton. The numbers of all species in the ring rooms were ten times lower than in the cardrooms. Counts for gram-positive species in the cardrooms of the mills spinning medium-grade cotton seemed low compared with counts in the other mills and resembled those in the mill using synthetic fiber, which were higher than in the mills spinning fine cotton. Moogan's results were not correlated with the prevalence of byssinosis in the mills because the questionnaires used by Cinkotai et al. (1988) were still being analyzed.

In 1984 results from one mill revealed that in some areas the number of bacteria per cubic meter of air fell during the shift. The air ventilation fans in this mill seemed more powerful, so figures for air change rates were obtained for 11 mills (D. Seaborn, personal communication 1985). The average air change rate for cardrooms was 10.8/hr (0 to 25 changes); for ring rooms it was 15.9/hr (10.6 to 23.0 changes), but the bacterial counts did not correlate with the air change rates in the ring rooms. Table 57 shows that the average airborne bacterial counts during a shift can vary quite a bit. The table presents results from two samples, taken 3 1/2 to 5 1/2 hr apart, in various areas of mills processing fine, medium, and coarse cottons. Airborne bacterial counts during single shifts of three mills that processed coarse cotton (with air change rates of 25.0, 8.3, and 3.0/hr) were analyzed statistically by the Kruskal-Wallis one-way analysis of variance test. Significant differences between the three mills were observed shortly after the shift

Table 57. Number of airborne bacteria during shifts in cotton mills that process fine, medium, or coarse cotton

	Airborne bacteria (hundreds of cfu/m³ air)											
	Fine cotton				Medium cotton				Coarse cotton			
	Card area		Ring area		Card area		Ring area		Card area		Ring area	
	Sample No.*		Sample No.		Sample No.		Sample No.		Sample No.		Sample No.	
Bacteria	1	2	1	2	1	2	1	2	1	2	1	2
B. brevis	1	0	0	0	3	6	1	2	12	0	1	2
B. coagulans	1	2	2	0	8	9	1	0	18	38	4	2
B. licheniformis	4	15	1	1	3	0	1	2	12	31	1	3
B. mycoides	9	20	2	1	3	0	0	0	4	1	4	5
B. polymyxa	15	24	3	4	17	15	3	3	58	61	8	7
B. pumilus/ B.subtilis	1	0	0	0	6	15	0	1	31	61	7	6
Total†	31	62	8	6	40	45	. 6	8	135	192	25	25
E. agglomerans	28	6	1	1	2	3	3	3	74	77	17	16
E. cloacae	0	0	1	1	8	3	1	0	6	30	1	2
P. cepacia	<1	1	0	0	0	6	1	0	12	46	3	4
P. maltophilia	0	0	0	0	0	0	<1	0	1	3	0	0
P. putida	0	0	0	0	<1	2	<1	<1	10	9	0	1
Total†	29	8	2	2	<10	14	5	3	104	165	21	23

*Time in minutes between samples 1 and 2 varied from 3 1/2 to about 5 1/2 hr.
†Includes other species not listed in this table.

Source: Moogan (1986).

commenced for *Enterobacter agglomerans* and four species of *Bacillus*; three other species achieved significant reduction by the end of the shift. Most of the *Bacillus* species and *Enterobacter agglomerans* were significantly reduced throughout the shift in the best ventilated mill. (Although many mills are air-conditioned, the level and consistency of the air-conditioning often varies.) Air change rates have not been considered previously, but these results suggest that more attention should be paid to this variable factor.

Air sampling (repeated three times per shift on 4 days during a 10-day period) in a mill that spun fine cotton revealed that the bacterial counts rose shortly after the machinery was started (Tuxford and Moogan 1986). The counts of all species in the cardrooms were lowest on the first samplings on the two Monday mornings and rose considerably during the shift. The greatest increases (tenfold in cardrooms on Mondays) were observed for *Bacillus polymyxa*, *Bacillus pumilus/Bacillus subtilis,* and *Enterobacter agglomerans*. Highest counts were recorded on Wednesday. Friday's counts, except for *Pseudomonas cepacia*, declined unexpectedly.

Samples of cottons and humidifier waters were also examined. *Bacillus* species were present in large

numbers (0.1 to 13.4 x 10^4/g) on the cotton, and counts were higher in mills that processed coarse grades of cotton. These mills also had higher counts of *Enterobacter* species (maximum of 22.0 x 10^4/g) and *Pseudomonas maltophilia* (1.0 x 10^4/g). *Pseudomonas putida* was present on samples of medium- and coarse-grade cottons. Very few bacteria were found on synthetic fibers. Spore-bearing bacterial species predominated in the water samples. Large numbers of *Bacillus polymyxa* (3.2 x 10^3/L) were isolated from the mill that processed fine-grade cotton and a synthetic fiber mill. *Enterobacter agglomerans* and *Enterobacter cloacae* were detected in the humidifier waters from the mills that processed medium-grade cotton and from one mill that processed coarse-grade cotton. Counts of species of *Pseudomonas* varied among the mills, and no *Nocardia* species were isolated from the humidifier waters.

Ninety-five claimants asking for compensation after contracting byssinosis donated sera for the detection of antibodies to bacteria isolated from the mills and to *Legionella* species. Slide agglutination was used to detect antibodies. In their blood sera, three claimants had antibodies to *Bacillus pumilus*, two had antibodies to *Bacillus mycoides*, one had antibodies to a *Nocardia* species, and two had antibodies to *Enterobacter agglomerans*. No serum had an antibody titre greater than 1 in 16 to any of these bacterial species. Three sera contained antibodies to *Legionella pneumophila* type 1 to titres of 1 in 16 detected by direct immunofluorescence. These titres were not considered high enough to warrant further investigation.

After confirming the identities of the bacteria isolated by Moogan (1986), Tuxford and Hoult (1988) screened 51 strains of the genus *Bacillus* and gram-negative species for cytopathic effect in Vero cell tissue culture and for toxin production by the Limulus lysate assay using the Coatest ELISA technique (by Kabi Diagnostics, Quadratech, P.O. Box 167, Epsom, Surrey County, England KT17 2SB). The ELISA technique was modified as recommended by the suppliers of the Coatest reagents for use in microtitre plates.

Two strains of *Bacillus pumilus* (M11 and M38), *Bacillus subtilis* (M67), *Bacillus cereus* (M27), and *Enterobacter agglomerans* (M14) were selected for further experiments. *Enterobacter agglomerans* and *Bacillus subtilis* had no effect on the Vero cells. *Bacillus cereus* had a different effect on the cells from that of *Bacillus pumilus*, which produced long fingerlike projections. Other effects were rounding and clumping of cells, followed by the development of holes and eventually detachment of the sheet.[9] Trypan blue was applied daily to determine the time of cell death (Jones and Schwab 1970). *Bacillus pumilus* M11 extract had a rapid effect that was cytotonic (greater than 50 percent rounded or affected cells), but the slower acting M38 supernatant was cytotoxic [death of greater than 50 percent of cells (Keusch and Donta 1975)]. The effects were reduced after boiling the bacterial extracts for 10 min and increased when the extracts stood at room temperature for 1 hr before centrifuging. In subsequent experiments, the supernatants were replaced with growth medium after standing at room temperature for 30 to 60 min; this caused regeneration of the cell sheets exposed to both of the strains of *Bacillus pumilus* (Hoult and Tuxford 1991).

Extracts of three *Flavobacterium* species, two *Enterobacter agglomerans* species, and six *Pseudomonas* species gave endotoxin readings with Coatest of 25 to 75 pg or greater than 75 pg. Two strains of *Bacillus cereus*, one strain of *Bacillus polymyxa*, and one strain of *Bacillus licheniformis* gave readings of 25 to 75 pg. *Bacillus megaterium* gave readings greater than 75 pg. The strains with high Coatest readings did not produce the greatest effects in tissue culture (table 58), so it was unlikely that lipopolysaccharide produced the cytopathic effects.

Effects of extracts prepared from 17 *Bacillus*, 4 *Pseudomonas*, 2 *Enterobacter,* and 2 *Flavobacterium* strains were tested on a cell line (A549) derived from human pulmonary epithelium (Hoult and Tuxford 1990). Undiluted *Bacillus pumilus* strains such as M11 and M38 produced the most

[9] Effects were graded in severity based on rounding and clumping of cells, development of cellular projections to produce a lacelike effect, holes in the cell sheet, and contraction and detachment of the cell sheet. Photographs of the effects are published in Hoult and Tuxford (1991).

Table 58. Cotton mill airborne bacteria, presence of lipopolysaccharide (LPS) as determined by ELISA Coatest, and cytopathic effects on Vero cells

Bacterial species	No. of strains*	Coatest LPS†	Cytopathic effect‡
B. brevis	3	v	v
	1	-	+
B. cereus	4	+	-
B. coagulans	5	v	+
B. firmus	1	v	-
	1	v	-
B. licheniformis	5	+	+
B. macerans	1	+	-
B. megaterium	2	+	v
B. polymyxa	1	+	-
B. pumilus	8	+	v
	3	v	v
B. subtilis	4	v	-
Flavobacterium	3	+	v
E. agglomerans	2	+	-
Pseudomonas spp.	4	+	-
	2	+	+

*Two samples of each bacterial strain were tested.
†+, Coatest LPS detected in both samples; -, no LPS detected in either sample; *v*, LPS detected in one of the two samples.
‡+, Both samples produced cytopathic effects (CPE's); -, neither sample produced CPE's; *v*, one sample produced CPE's.
Source: Tuxford and Hoult (1988).

severe effects, causing contraction or destruction of the cell sheets within 10 min. One strain of *Bacillus licheniformis* (M47) caused contraction within 10 min. The other produced holes in the sheet, with a lacelike effect developing by 20 hr. Although both strains of *Enterobacter agglomerans* produced minimal effects in 10 min, the M14 extract produced grade-three lacelike effects in cells 20 hr after treatment. Similar results were observed for one *Flavobacterium* species and *Pseudomonas putida*; one *Pseudomonas* species caused the cell sheet to contract within 20 hr. When the extracts were diluted, the effects were generally reduced. This reduction occurred especially for the extracts from gram-negative species. For some species, however, reduction did not occur. For example, at the one-eighth dilution, *Bacillus licheniformis* (M47) and *Bacillus pumilus* (M11) still caused contraction of the cell within 10 min.

During 1988 sera from 12 Danish cotton workers were tested for antibodies using an enzyme-linked immunosorbent assay on 3 species of *Bacillus* and 1 strain of *Enterobacter agglomerans* isolated from the air of Lancashire cotton mills. Six sera were from workers with byssinotic symptoms and six were controls, that is, they were from workers who did not have byssinosis and who were of the same occupation, age, and gender as the other six (Tuxford et al. 1989). Antibodies were detected (table 59), but there was no pattern distinguishing the sera of those with byssinosis from the control sera.[10] Although the sera were absorbed, cross

Table 59. Antibodies to *Bacillus* and *Enterobacter* species in sera of 12 Danish cotton workers measured by ELISA

	Number of sera containing antibodies				
				B. pumilus	
ELISA*	*E. agglomerans*	*B. cereus*	*B. subtilis*	M11	M38
+	5	4	5	5	5
v	3	4	5	5	0
-	4	4	2	2	7

*Two samples were tested. +, The readings of both ELISA tests were greater than the mean of readings for the group of cotton workers' sera tested in duplicate. *v*, The reading for one test was above the mean reading of the group and for the other test was below. -, The readings from both tests were less than the mean reading of the group.
Source: Tuxford et al. (1989).

reactions probably occurred. It was very interesting and probably significant that Danish cotton workers had antibodies to English strains of bacterial species to which they had never been exposed but that had been isolated from similar workplaces in another country.

Comparisons of the bacterial contents of cotton, flax, hemp, and jute were made by Nicholls et al. (1991). Except for a single isolate of *Pseudomonas aeruginosa* from one cotton sample, only gram-positive species were isolated from the cotton and jute samples, with *Corynebacterium* species predominating on the cotton. The most numerous species of *Bacillus* were pigmented and nonpigmented strains of *Bacillus pumilus*. Details of the other species are shown in table 60.

Six spinning mills for processing cotton and two for processing man-made fibers were surveyed by Niven et al. (1991). Air samples were collected with an Anderson particle fractionating sampler. Plates from stages 3 to 6 of the sampler were combined to give the total level of respirable airborne microorganisms. Nutrient agar incubated at 25 °C and 37 °C was used to select for gram-positive bacteria (the medium itself, however, was not a selective medium), violet red bile glucose agar at 37 °C was used to select for GNB[11], tryptone soy agar at 52 °C was used to select for thermophiles, and malt agar at 25 °C and 37 °C was used to select for fungi. All cultures were incubated 72 hr for maximum growth.

The counts of viable airborne microorganisms are shown in table 61. Multiple regression analysis (Lotus 123) showed a significant relationship between byssinosis and gram-positive bacteria ($p < 0.001$) and also byssinosis and GNB ($p < 0.05$). No significant correlation (Pearson equation) existed between dust in the work area and gram-positive bacteria. Personal dust samples with these bacteria gave a Pearson coefficient of 0.60. These results further supported the hypothesis that airborne bacteria, especially gram-positive species, may be of importance in the etiology of byssinosis.

Table 60. Gram-positive bacteria on three different cotton samples

	Gram-positive bacteria (thousands of cfu/g)		
	Cotton sample numbers		
Bacteria	1	2	3
Total *Bacillus* spp.	391	219	369
pumilus pigmented	219	104	276
pumilus nonpigmented	160	92	69
Corynebacterium spp.	620	805	736
Micrococcus spp.	24	92	58
Actinomyces spp.	23	0	23
Total (all species)	1,058	1,116	1,186

Source: Nicholls et al. (1991).

Discussion

A comprehensive review of the literature on byssinosis was published by the Shirley Institute, Manchester, England (Honeybourne et al. 1982). The number of new cases of byssinosis in the United Kingdom is declining (Fishwick et al. 1990) despite less stringent qualifications for compensation and changes in criteria for diagnosis (Schilling et al. 1952, Rylander et al. 1987, Elwood 1988). At least 1 yr has occurred when no new cases were reported in the industry (Shelmerdine, personal communication 1988). The population at risk in Lancashire has also changed both in ethnic origin and in the number of people employed.

Measures taken by the industry to improve working conditions may have reduced the prevalence of byssinosis. Air-conditioning has reduced dust but has not controlled the disease, since byssinosis has occurred in air-conditioned mills (Cinkotai, personal communication 1986). Surveys by Winch and Tuxford (unpublished data) have shown that maintenance, especially of filters, is not always perfect. Moogan (1986) found unexpectedly large differences in air change rates within mills and found that these rates did not correlate with bacterial counts;

[10] The technique used by Tuxford et al. (1989) was more sensitive than that used by Moogan (1986).

[11] Baltimore Biological Laboratories (1968, p. 155) stated that bile salts and crystal violet will inhibit most but not all gram-positive organisms.

however, he made no assessment of the effects of the filters in the mills.

Very few of the studies mentioned in this chapter were conducted with the advice of a professional statistician. Statisticians and the staff of the site to be surveyed should be consulted during the design stage of any survey in which the researcher wants to obtain accurate results. Liaison with the site staff should ensure that test conditions represent normal working practices and that surveys cause minimal disruption to work schedules.

Although attempts have been made to reproduce some of the microbiological surveys described in this chapter, the task is difficult because considerable variation in microbe counts occur within shifts from hour to hour and day to day. In addition the microbial flora on cotton varies. The media used affects which microbial species are likely to be detected. If a different (for example, richer) medium is used, other microbial species might be detected. Comparisons among existing surveys are difficult because these surveys used different media, temperatures, and durations of incubation.

Variation in the composition of the microbial flora detected is also influenced by the experience of the bacteriologist. Identification of *Bacillus* species may be difficult. The gram stain often gives misleading results, especially on an old culture. Sporulation should be sought before the gram stain is perfomed, or extremely young cultures should be stained. The stain must be interpreted correctly before the API system is used; otherwise gram-positive *Bacillus* species may be identified as members of the *Enterobacteriaceae*. For example, *Bacillus pumilus* will appear to be *Enterobacter agglomerans* (Salkin, personal communication 1988). Not enough is known about the ability of *Bacillus* species to grow on media designed to select *Enterobacteriaceae*. Some species grow in the presence of bile salts and may then stain as if they were gram-negative species (Furness 1951; Tuxford, personal observation 1985). Names of species may be changed as additional information is added to the database. Important criteria for identification have changed between different editions of textbooks.

A puzzling discrepancy in the research discussed here is the failure of Tuffnell (1960) to find any GNB. Details of his identification techniques are not available, but he should not have missed easily recognizable gram-negative species known from earlier work to be present in cotton dust (Furness 1951). His work was confined to cardrooms, and it is possible that gram-negative species were present in the air in such relatively small numbers that they were not detected. Furness did not detect GNB on his gram-stained films, but this may be due to his

Table 61. Viable airborne microorganisms in four ring and two open-end cotton spinning mills

		Microorganism count (thousands of cfu/m³)		
Mill type and area	Airborne organisms	High	Low	Mean
Ring Mills:				
Opening, blowing, and carding area	Gram-positive bacteria	88.4	27.2	52.1
	GNB	8.0	0.3	2.4
	Thermophiles	7.1	0.7	3.3
	Fungi	34.7	6.1	17.3
Ring, spinning area	Gram-positive bacteria	3.4	1.4	2.3
	GNB	0.1	0.0	<0.1
	Thermophiles	0.3	0.2	0.2
	Fungi	0.8	0.2	0.5
Open/End Mills:				
Open/end, spinning area	Gram-positive bacteria	30.8	18.0	24.4
	GNB	0.9	0.3	0.6
	Thermophiles	1.0	0.8	0.9
	Fungi	6.8	2.9	4.9

Source: Niven et al. (1991).

use of petroleum-ether to remove oil from the dust (Furness and Maitland 1952). An alternative hypothesis is that many *Enterobacteriaceae* isolated by other researchers were members of the genus *Bacillus* that stained as if they were gram-negative or grew on the media selective for gram-negative species. Nicholson (1980) considered that he had excluded these possibilities. The latest English study to examine cotton fiber (Nicholls et al. 1991) supported Tuffnell's (1960) findings.

Toxins of *Bacillus* species producing cytopathic effects in tissue culture (Tuxford and Hoult 1988) need to be isolated and identified. In the only surveys to study free endotoxin in the Lancashire cotton mills, samples were sent to Sweden for analysis (Cinkotai et al. 1977). It is not known whether adequate precautions were taken to prevent contamination with extraneous endotoxin. Some gram-positive bacteria were positive in a Limulus lysate assay (Tuxford and Hoult 1988), suggesting that a possible source of error might occur when measuring endotoxin levels from GNB in air samples from the cotton mills.

Species isolated from the Lancashire mills included several members of the genera *Bacillus* and *Pseudomonas* and the family Enterobacteriaceae (including several strains of *Enterobacter agglomerans*) and included some *Nocardia* species, micrococci, and occasional corynebacteria. Surprisingly few corynebacteria were detected in older studies. This may be due to a lack of knowledge about plant bacteria in medically oriented departments. The fungal flora is complex but is dominated by *Cladosporum*, *Penicillium,* and *Aspergillus* (Lacey and Lacey 1987).

Viable counts of airborne bacteria in mills diminish as raw cotton is turned into yarn, and ring rooms have at least 10 times fewer bacteria than cardrooms. In English cotton spinning mills viable counts in general have diminished over the years. This decrease may be due to the annual alteration from year to year of the microbial flora on the cotton, but it also has been attributed to better ventilation. Alterations in the numbers of viable organisms throughout the workshifts should be further investigated. Machinery startup increases the number of microorganisms in the atmosphere (Tuxford and Moogan 1986). The ventilation rates and state of the air-conditioning should be examined to determine whether either significantly affects the bacterial counts.

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Chapter 13. Commercial Cotton Mills: A Changing Environment

Janet J. Fischer and Karin K. Foarde

Many factors that are impossible to quantify accurately have combined to reduce the incidence of byssinosis in workers in the cotton industry in the United States and the United Kingdom from 51 percent in earlier years (Roach and Shilling 1960) to 10 percent in 1989. One factor in the United States has been Federal mandates to keep airborne dust under control in the cotton industry. The federally mandated cotton-dust limit is 200 mg/m^3 for air in which employees are working an 8-hr workshift and 134 mg/m^3 for air in which employees are working a 12-hr shift (Fletcher et al. 1990, Domelsmith et al. 1991).

This chapter documents the changes in dust levels and airborne levels of gram-negative bacteria (GNB) and endotoxin [measured as lipopolysaccharide (LPS), the active component of endotoxin] that have occurred in a series of mills followed over a period of years. The levels of GNB and endotoxin in the cotton coming into the mill varied from year to year, depending on the source of the cotton and the cultivating and harvesting conditions each year. To help explain some of the variability in the data on the levels of dust, GNB, and endotoxin, we have included data on the quality of the cottons processed, the type of blend used in the individual mills, the season of processing (winter or summer), and whether the carding area is separate from the spinning area. Because cotton processing involves multiple stages, data from spinning have also been included.

Over the last decade multiple changes have been made in the cotton mills of North Carolina. Engineering improvements have had a profound effect on the numbers of microorganisms associated with cotton processing in the mills (Fischer et al. 1992).

With the cooperation of industry, we studied the mill environment and how it is affected by the cotton processed. These data were supplemented by a few studies of independent mills and some ongoing studies in the model cardroom at the School of Textiles at North Carolina State University (NCSU). A wide variety of factors were studied. These included levels of microorganisms (gram-negative bacilli, gram-positive bacilli, fungi, corynebacteria), endotoxin, and dust. Much has been written about dust levels in the mills, but very few studies correlate those levels with counts of viable bacteria. It is known that dust is heterogeneous and that the dust from one mill may be quite different from that from another mill. The Federally mandated cotton dust standard (or limit) applies to dust on a weight basis.

This paper presents an overview of the data collected and explores the relationships between variables. In our studies cotton samples were obtained from various places in the cotton-processing line and from bales of cotton in the opening room of commercial cotton mills. Airborne cotton dust samples were obtained by vertical elutriator (VE) filters. All specimens were processed promptly in the same laboratory. Dust levels in our earlier studies were determined by weighing VE filters or later by PCAM measurements. [PCAM is a portable single sensor version of the multisensor Continuous Aerosol Monitor (CAM) system.] The PCAM system's electro-optical measure of dust (airborne) is equivalent to the amount of dust that would be collected (and measured) on a VE filter, the device used in the standard method of measuring airborne dust (Shofner et al. 1981). Filters, cotton specimens, and dusts were extracted in water and studied for content of viable bacteria (gram-positive and gram-negative), fungi, and endotoxin by methods described previously (Fischer et al. 1989).

Our Early Studies

Our early studies (in the research laboratory of Janet J. Fischer at the University of North Carolina) show that levels of airborne gram-negative rods and endotoxin correlate roughly with VE dust levels in model cardrooms and in the mills and are lower in the spinning area than in the carding area (Fischer 1979, 1980). Steaming of cotton for 1 min results in a 2 log decrease in counts of GNB in the lint and greater than a 1 log decrease in the counts of airborne GNB (Fischer 1979).

Few types of GNB are consistently present in the air of cotton mills, in cotton of various cultivars, in cottons from foreign countries, and on the different parts of the cotton plant (Fischer and Kylberg

1983). The two most prominent organisms are *Enterobacter agglomerans* and *Pseudomonas syringae*. *Flavimonas oryzihabitans* is also commonly found but had not been identified as a separate species in the early eighties; instead it was grouped with *Pseudomonas* species. Levels of GNB and endotoxin are similar whether obtained from VE or PCAM filters (Fischer and Thomas 1983).

The following conclusions were established from previous work in our laboratory:

1. Marked changes in the bacterial counts of lints are associated with lesser changes in the bacterial counts of airborne dusts (Fischer 1979, 1980; Fischer and Thomas 1983).
2. Airborne endotoxin levels correlate roughly with the numbers of airborne GNB (Fischer 1979, 1980; Fischer and Thomas 1983). Correlation coefficients established the significance of this relationship for the carding and spinning areas of mills in 1978–1979, and three different groups of investigators reaffirmed this correlation in 1982.
3. The use of an oil overspray decreases airborne dust, airborne GNB, and airborne LPS. This was true in the model cardroom at NCSU and in the mills. Apparently, the oil holds the microorganisms on the cotton so that the number of organisms becoming airborne is decreased not just in the early stages of the process but all the way down the cotton-processing line (for example, down to the spinning area). Although fewer GNB become airborne when oil is used, the same percentage of those becoming airborne reach the alveoli as if oil was not used (Fischer and Witcher 1984). The same is true of endotoxin. We found no evidence of a shift of the liberation of GNB or LPS further down the cotton-processing line.
4. Heavy rains in the southern United States during harvest in 1984 caused the grade of the cotton crop to deteriorate (Fischer and Sasser 1987). The levels of GNB, corynebacteria, LPS, and fungi in the 1984 crop were markedly higher than in the 1983 crop (Fischer et al. 1989).
5. The ratio of LPS (in nanograms) to dust (in micrograms) is a useful way to compare environments. The ratio is less than 0.1 for mills processing synthetic fibers and 0.40 to 1.0 in the carding area for mills processing cotton. The ratio is much lower in spinning areas (Fischer et al. 1990).
6. Airborne dust and LPS levels in mills do not change much as long as the quality of the cotton to be processed remains the same. Studies of three independent mills confirmed the constancy of the airborne dust and LPS levels over a 1- to 2-wk period (Fischer et al. 1990).

Mill Studies by Other Investigators

Other workers have also looked at the mill environment. Many studies by many different researchers have been done on the Lancashire textile mills, and these studies are summarized by A.F. Tuxford in chapter 12. When Tuxford and Moogan sampled the mills between 1983 and 1985, they isolated many species of *Bacillus* and also *Enterobacter agglomerans*, *Enterobacter cloacae*, and *Pseudomonas cepacia*. Quantitative studies of airborne bacteria revealed that the levels of *B. polymyxa* and *B. subtilis* were usually higher than the levels of *Enterobacter agglomerans* (the most numerous of the species of gram-negative rods) (Tuxford and Moogan 1986).

Another group of investigators documented endotoxin exposure levels of 3,600 ng/m^3 in the cotton-opening rooms and less than 30 ng/m^3 in mills processing man-made fibers. They found a high correlation between byssinosis and endotoxin levels (r=0.51) and some correlation of endotoxin with respiratory symptoms (r=0.38). Means of measured levels of endotoxin in cotton dust were 524 ng/mg in conventional cotton mills, 138 ng/mg in mechanized cotton mills, and 37 ng/mg in mills processing man-made fibers. These means contrasted with means of measured levels of personal endotoxin exposure of 898, 182, and 11 ng/m^3, respectively, in these same mills (Niven et al. 1992).

These same investigators looked at the airborne microorganisms and determined coefficients of correlation between the microorganisms and workers with byssinosis, workers with respiratory symptoms, and workers with chronic bronchitis (table 62). There were no significant correlations between workers with chronic bronchitis and the concentra-

Table 62. Pearson coefficients of correlation of workers with byssinosis, symptoms in general, and chronic bronchitis with levels of airborne microorganisms or dust

Microorganisms and dust levels	Pearson coefficients (p values in parentheses)*		
	Byssinosis	Symptoms	Chronic bronchitis
Microorganisms			
Gram-positive bacteria	0.76 (<0.001)	0.56 (<0.001)	.33 ns
GNB	0.44 (<0.05)	0.07 ns	.31 ns
Fungi	0.60 (<0.001)	0.29 ns	.27 ns
Thermophilic bacteria	0.60 (<0.001)	0.52 (<0.001)	.21 ns
Dust level			
Area dust level	0.34 (<0.05)	0.14 ns	.13 ns
Personal dust level	0.52 (<0.001)	0.22 ns	.27 ns

*ns, Not significant.

Source: Adapted from Niven et al. (1991).

tion of microorganisms or dust. The correlations with byssinosis were highest for gram-positive organisms but also were significant for gram-negative organisms (Niven et al. 1991). Since the gram-positive bacterial content of cotton remains stable much longer than the gram-negative bacterial content of cotton during storage, one would expect byssinosis to correlate better with gram-positive bacteria.

A 5-yr study of cotton textile workers in six textile plants (four that processed cotton and two that processed synthetic fibers) in North Carolina was begun in 1982 by Tulane University personnel. Environmental measurements, for example, dust levels, bacterial counts, and endotoxin levels, were associated with individual workers according to their plant, work area, and workshift. Pulmonary function and environmental measurements were made a week apart. The environmental indices all highly correlated with each other. The correlation coefficient for log dust level with log endotoxin was 0.78. The correlation coefficient for log dust level with log bacterial counts was 0.85, and the coefficient for log endotoxin level with log bacterial counts was 0.92. The high correlations between the environmental indices were confirmed by three different groups of investigators but were only measured during the first year of the study.

The only pulmonary function data that significantly correlated with the logarithm of the dust level was forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC). CAM's were used to measure dust levels and vertical elutriators were used to gather dust for the microbiological assays (Diem et al. 1984).

In the first year of the study it was concluded that pulmonary function correlates (negatively) only with the log of the dust level. In subsequent years environmental indices were not measured. Rylander and Haglind (1983) found that pulmonary function did not correlate with dust level but strongly correlated with endotoxin level. Our previous work indicates that these relationships are difficult to establish. In 1978–79 we found good correlations (Spearman coefficient method) between FEV_1 data and airborne endotoxin levels in the carding area of mills. When the same mills were resampled (second biannual sampling) during the summer, however, we found no correlation (Fischer 1980).

The average elutriated dust concentrations in the mills surveyed in North Carolina were 0.069 to 0.396 mg/m^3 (first study year), 0.089 to 0.391 mg/m^3 (second study year), and 0.106 to 0.210 mg/m^3 (third study year). The third-year data are at or below the mandated Federal standard for maximum allowable dust (Abdel-Kader et al. 1987).

Another report from this 5-yr study showed a significant dose-response relationship in cotton yarn manufacturing between annual declines in FEV_1, FVC, and forced expiratory flow (25–75 percent) and average dust exposure by mill. The larger declines were found in mills using the highest percentage and lowest grade of cotton (Glindmeyer et al. 1991).

The reports from the 5-yr study do not provide data on the microbiological content of the dust or the air. Diem et al. (1984) discussed log counts of gram-negative rods and log endotoxin level only for the first year of the study and concluded that the most important environmental index affecting pulmonary function is log dust level. Our study results, however, are in agreement with those of Rylander and Haglind (1983), which show that endotoxin level of the air correlates negatively with pulmonary function.

A final conclusion from the 5-yr study was that the mill work environment is detrimental to the pulmonary function of workers who smoke (Glindmeyer 1993).

Our Studies of the Mill Environment Over a 5-Yr Period

We also performed a 5-yr study on the environment of commercial mills (samples studied in the research laboratory of Janet J. Fischer at the University of North Carolina). In this study the mills were placed into one of two categories: those processing less than 50 percent cotton and those processing more than 50 percent cotton. Some of the mills had separate rooms for carding and spinning, whereas some combined these two functions in one area. During the 5 yr, the air quality in the mill generally improved. The improvement in the air quality (decrease in airborne levels of GNB, LPS, and dust) was more obvious over time when carding and spinning were separate and when mills processed less than 50 percent cotton (Fischer et al. 1992). The data from this study are summarized here, and the tables in this chapter were adapted from this study.

Tables 63 and 64 list mills in which carding and spinning are located together. The mills mentioned in table 63 used a blend containing more than 50 percent cotton, and those mentioned in table 64 used a blend containing less than 50 percent cotton.

Table 63. Bacteria, endotoxin (measured as LPS), and dust levels in mills in which carding and spinning were located together and a blend containing more than 50 percent cotton was processed

		GNB (cfu/m^3)		Endotoxin (LPS, in ng/m^3)		Dust (mg/m^3)	
Mill No.	Year	Carding	Spinning	Carding	Spinning	Carding	Spinning
2	78–79	2,800	3,800	2	0.2	322	228
	79–80	9,400	2,600	112	11	173	219
	80–81	7,300	9,800	27	28	218	290
	81–82	8,900	3,000	284	29	237	438
	82–83	2,798	349	388	220	151	125
3	78–79	1,500	650	21	1.2	216	336
	79–80	7,000	2,500	110	11	208	214
	80–81	55,000	2,500	1,305	14	485	322
	81–82	11,000	1,500	280	2,793	301	298
	82–83	1,050	500	379	233	206	269

Source: Fischer et al. (1990).

During the 5-yr period GNB levels decreased (but much more in mills that processed blends of less than 50 percent cotton, table 64). Endotoxin (LPS) levels increased but the actual values in mills that processed blends of more than 50 percent cotton were at least double those in mills that processed blends of less than 50 percent cotton. Dust levels did not decrease much, if at all, over the 5-yr period.

Tables 65 and 66 list mills in which carding and spinning are located in separate areas. Again, GNB levels decreased and endotoxin (LPS) levels increased over the 5-yr study. This is especially noticeable in mills that processed blends of more than 50 percent cotton (table 65). Dust levels are extremely variable because cotton quality, source of the cotton (area where grown), and weather and harvesting conditions vary from year to year. Dust levels decreased during the 5-yr period. Similar data (not reported here) were obtained from other cotton mills.

Dust was kept under control (that is, under the federally mandated dust level of 200 mg/m^3) when carding and spinning were located in the same area and when a blend containing less than 50 percent cotton was carded (table 64). If a blend containing more than 50 percent cotton was carded, the dust was likely to approach or exceed the federally mandated maximum level (table 63, 1983 data).

In mills that process blends of more than 50 percent cotton, the dust levels were similar for carding and spinning, whether these functions were located in the same area or in separate areas (tables 63 and 65). However, in mills that process blends of less than 50 percent cotton (California cotton), the dust levels in spinning were lower than in carding when these functions were located in separate areas (table 66).

The endotoxin data varied a lot presumably because of variations in methodology. Endotoxin levels were low in mills that had carding and spinning located in separate areas and that processed blends of less than 50 percent cotton.

If LPS (endotoxin) is the agent precipitating respiratory symptoms, FEV_1 should not have declined much over the 5-yr period. But symptoms may be unreliable, and unfortunately not enough data on FEV_1 was available to establish a reliable correlation coefficient between LPS and FEV_1, so the question cannot be answered.

Table 64. Bacteria, endotoxin (measured as LPS), and dust levels in mills in which carding and spinning were located together and a blend containing less than 50 percent cotton was processed

		GNB (cfu/m^3)		Endotoxin (LPS, in ng/m^3)		Dust (mg/m^3)	
Mill No.	Year	Carding	Spinning	Carding	Spinning	Carding	Spinning
5	78–79	1,900	1,500	1.5	1.5	86	136
	79–80	2,300	1,000	56	6	133	275
	80–81	3,400	640	14	14	78	79
	81–82	4,000	1,500	141	14	82	91
	82–83	300	20	135	136	58	52
6	78–79	6,800	580	1.5	1.4	125	197
	79–80	9,100	3,900	110	11	147	140
	80–81	1,200	1,400	3.3	14	141	197
	81–82	1,600	20	195	73	129	138
	82–83	813	91	103	84	159	145

Source: Fischer et al. (1990).

Table 65. Bacteria, endotoxin (measured as LPS), and dust levels in mills in which carding and spinning were located separately and a blend containing more than 50 percent cotton was processed

		GNB (cfu/m³)		Endotoxin (LPS, in ng/m³)		Dust (mg/m³)	
Mill No.	Year	Carding	Spinning	Carding	Spinning	Carding	Spinning
10	78–79	1,200	1,500	1.8	0.2	253	181
	79–80	3,800	1,100	113	52	141	167
	80–81	8,800	440	27	27	153	145
	81–82	450	<280	3	28	46	102
	82–83	22	4.8	336	192	11	71
12	78–79	10,000	880	23	2.3	532	172
	79–80	15,000	2,400	75	75	312	449
	80–81	34,000	3,700	282	169	407	284
	81–82	12,000	2,200	288	283	632	209
	82–83	177	16	327	316	68	133

Source: Fischer et al. (1990).

Table 66. Bacteria, endotoxin (measured as LPS), and dust levels in mills in which carding and spinning were located separately and a blend containing less than 50 percent cotton (California cotton) was processed

		GNB (cfu/m³)		Endotoxin (LPS, in ng/m³)		Dust (mg/m³)	
Mill No.	Year	Carding	Spinning	Carding	Spinning	Carding	Spinning
20	78–79	880	<75	1.4	<0.2	246	238
	79–80	230	<29	38	4	159	91
	80–81	130	<7	14	7	211	96
	81–82	140	<19	7	0.7	177	46
	82–83	<1.2	6.5	59	4	92	16
21	78–79	600	<84	1.4	0.2	555	104
	79–80	690	<22	54	5	234	130
	80–81	<28	<24	2.8	2.4	205	63
	81–82	280	<3.4	17	--	249	48
	82–83	76	2.0	113	5	128	27

Source: Fischer et al. (1990).

The above data serve as an important baseline for current studies on the textile mill environment.

Summary

As shown by our studies in commercial mills, the levels of dust and airborne GNB in cotton yarn manufacturing mills has improved over the past few years. Levels of endotoxin, however, appeared to increase during the study period. Unfortunately the endotoxin data from 1 yr to the next are not comparable because the method used to obtain the data was changed during this period of study, and the newer method of endotoxin testing had increased sensitivity.

Some disagreement exists about which of these factors—dust, GNB, and endotoxin—is the most important in the commercial mill. Chapter 16 of this book presents studies of the pulmonary function of cotton workers and volunteers not previously exposed to airborne materials (until they were exposed to them in a model cardroom), and these studies implicate endotoxin as a major (but perhaps not the only) factor in the etiology of byssinosis. Although the incidence of disease is decreasing as the mill environment improves, mill exposure, especially for smokers, still causes a decrease in pulmonary function.

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Chapter 14. The Model Cardroom as a Research Tool

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Raw cotton is received at the textile mill in highly compressed bales that contain varying amounts of trash and dust. In the yarn-manufacturing process, the compressed stock is progressively opened and reduced to smaller and smaller fiber tufts as it moves through the opening machines (Hamby 1965). During this process, large trash particles and coarse dust are removed. However, some fine trash and much of the microdust and respirable dust remain in the fiber batt that is prepared for introduction to the card. (Trash and dust particle sizes defined by ITMF (1984) are as follows: trash, ≥ 500 μm; dust, 50 to 500 μm; microdust, 15 to 50 μm; respirable dust, ≤ 15 μm.)

Carding is the most important process in the preparation of raw cotton for spinning. The fibers are individualized and assembled into a thin web, providing a stock that is ideally suited for subsequent controlled reassembly into yarn. Microdust and respirable dust generation is maximized during carding, since the fibers are at a maximum state of openness and vigorous mechanical and pneumatic forces are applied to the fiber. Thus, carding is the logical, ideal operation for controlled study of cotton dust. Additionally, the carding machine can be isolated from the other machines and areas of the yarn-manufacturing process without compromising the operation as an industrial process.

Model cardrooms have been an effective tool for facilitating research on the factors associated with the generation of cotton dust and on the relationships between the physical, chemical, and microbiological characteristics of cotton dust and the incidence of byssinosis. One of the first model cardrooms was used by Merchant et al. (1973) to study the acute stages of byssinosis under controlled conditions and to evaluate the effectiveness of preprocessing treatments such as steaming to "deactivate" cotton dust as a byssinosis causative agent. No attempt will be made to describe all possible carding systems used for research purposes. Instead we will concentrate on the model cardroom at the U.S. Department of Agriculture, Agricultural Research Service (USDA–ARS), Cotton Quality Research Station (CQRS), Clemson, SC, because of its extensive use and our familiarity with it. Cocke and colleagues have described this cardroom elsewhere (Cocke 1981a, 1982; Cocke and Bargeron 1984, 1985; Cocke et al. 1975; Harrison and Cocke 1984a). This chapter consolidates their detailed descriptions.

Description of the USDA–ARS Model Cardroom

The USDA–ARS cardroom was designed as a test facility as part of the comprehensive research program initiated by the USDA–ARS Cotton Quality Research Station in the early 1970's. The program was designed to study various aspects of cotton dust associated with cottons produced in the United States. Early research generally focused on (1) determining variations in dust levels related to variety, area of growth, and grade and (2) determining the extent that dust levels could be controlled by mechanical cleaning during ginning and the early stages of textile processing. This research was in response to the Occupational Safety and Health Administration's (OSHA) 1971 standard that limited cotton dust levels in yarn manufacturing facilities to 1,000 $\mu g/m^3$ as measured by a personal sampler. Many mills could not comply with this standard because of limited air handling and filtration systems, open machines that allowed unrestricted liberation of dust, and a general lack of knowledge of the dust generation potential of cotton and the factors in mill processing that affect dust levels.

Later research focused on (1) determining the effects on dust levels of carding parameters such as production rate, the amount of air used in the on-card cleaning system, and the number of air changes per hour, (2) controlling dust levels by treatments with additives during harvesting, ginning, or textile processing, (3) characterizing dust by particle size and size distribution, (4) determining levels of microorganisms and endotoxin in dusts from cottons of diverse histories, (5) deactivating or removing biological activity of dust by washing raw cotton with water, and (6) identifying causative agents of byssinosis. Many of the latter projects involved the use of human panels to determine effects of dust levels and dust composition on acute pulmonary response.

This research was conducted in a facility designed and constructed to generate cotton dust (comparable to that generated in mills) under highly controlled conditions. Initially, two identical rooms were built. Each contained a standard lap-fed Saco Lowell card, capable of production speeds up to 45 kg/hr, and the required test equipment and instrumentation. The rooms were completely enclosed and isolated for independent operation. Adequate space was provided for movement of personnel and equipment around the card during operation and for seating up to about 20 human panel members when required. Each room was approximately 6.25 m long, 4.28 m wide, and 3.05 m high, which amounts to a volume of about 81.6 m^3.

An air exhaust system with a fan and filtration unit capable of moving from 0 to 39 m^3 of air per minute is located outside the room. Air pickup points on the card are located where dust concentrations are normally the highest to ensure maximum flexibility for dust control. The supply air comes from within an adjoining part of the building where conditions are maintained at 24.0 °C and 55 percent relative humidity. The supply air passes through a high-efficiency composition filter and an electrostatic precipitator. Temperature is maintained by a chilled water coil and a modulated steam heating coil. Located within the air inlet ducting system is a modulated steam-humidifying system that controls air humidity. The effect of air currents on the uniformity of dust generation by the card is minimized by locating the supply air vents near the ceiling. The supply air can be adjusted from 0 to approximately 62 m^3/min by a valve located in the ducting system so that as many as 45 room air changes per hour can be achieved. Although conditions can be varied, for standard test conditions 11.5 room air changes per hour (15.6 m^3/min) at 24.0 °C and 55 percent relative humidity are generally used. The temperature and humidity conditions are controlled by sensors located in the room.

The air supply and exhaust system can be operated as a balanced system or as a positive-pressure system. In the balanced mode, the volume of air exhausted through the card-cleaning system is equal to the volume of air supplied. In the positive-pressure mode, the volume of supply air is in excess of the volume of exhaust air. The excess air is forced out through a venting system near the floor on the opposite side of the room from the point of entry. This exhaust duct can be equipped with a filter screen that can remove fibers and large trash particles. A portable electrostatic precipitator can be attached to the end of the exhaust duct to collect the remaining particles. If dust in excess of that collected on filter media is required for analysis, the dust on the precipitator collector plates can be carefully removed and used.

Addition of Exposure Chamber

The model cardroom has been used for more than just generating, measuring, and controlling dust levels produced by cotton during the carding process. As mentioned, the room is large enough to serve as an exposure chamber for human panels or small animals. However, practical and physical considerations come into play for extended human studies. The size of the room poses a practical limit on the number of people who can participate in a study. Movement of plant personnel responsible for operating the card and instrumentation is hampered when test subjects are in the room. When more than 20 people are placed in the room, the capacity of the cooling system is compromised, and standard temperature and humidity conditions are difficult to maintain.

Within the room, air currents created by the card and release of dust from high concentration points on the card result in different but consistent dust levels at the four sampling locations. This necessitates rotation of panel members about the room during the exposure period. Further, because the card is a full-scale commercial card, safety is always a consideration even though guards and rails are in place around the card and rigid safety rules are enforced. Also, for most subjects the noise levels, although not beyond recommended levels, are an irritant. To fully compensate for the noise and safety concerns, a facility was designed as a necessary extension and adjunct to the model cardroom.

Three modular units, each 9.14 by 7.32 by 2.44 m (length by width by height), were purchased. Initially, two of the units were used as dust exposure chambers and the third served as the control center. Each modular unit is equipped with a heat pump,

and conditions within each unit are maintained at 24.0 °C and 55 percent relative humidity. The units are located adjacent to but totally isolated from the permanent facilities of the operating pilot plant. The units have entrances separate from the pilot plant and access can be gained directly from the parking area. Each of the exposure chambers can be supplied with dust-laden air from the model cardrooms.

The module serving as the control center unit is equipped with a waiting room, rest room facilities, storage areas, and equipment and instrumentation for lung function testing, including two spirometers (each housed in a separate room) and an automated transmural-breathing pressure/volume plethysmograph. Space for additional equipment is available as needed.

The two exposure units are spacious enough to house as many as 30 to 35 panel members and all dust-sampling equipment. To provide for comfort, the units are equipped with tables, chairs, cold-water dispensers, a hot-water dispenser for making soups and hot drinks, a two-section sink for washing eating utensils, and a soft drink dispenser. The general procedure for a panel member is to first report to the control center for pre-exposure lung function tests and then go to the assigned dust exposure chamber for a minimum 6-hr exposure. The panel member must stay in the exposure chamber until the conclusion of the exposure and then return to the control center for postexposure lung function tests. The only other time the panel member is permitted to leave the exposure chamber is to use the rest room in the control center. These facilities in modified form are still in place at the Cotton Quality Research Station (CQRS).

Dust-Sampling Systems

Six types of dust-sampling systems are available for dust sampling in each cardroom and in each remote dust exposure chamber to determine dust levels and to collect dust for physical, chemical, and biological characterization. The vertical elutriator (VE) is the dust sampler specified by the OSHA Cotton Dust Standard (Federal Register 1978, 1985). The VE sampler is designed to capture particles whose aerodynamic diameter is 15 μm or less when used with an upward air flow rate of 7.4 L/min. Typically, four VE samplers are used in each cardroom and in each dust exposure chamber. Each VE sampler is located at a designated sampling station that is connected to a high-vacuum pump by a vacuum line. Each cardroom and exposure chamber is serviced by separate high-vacuum pumps located outside the room or chamber. In the exposure chambers, the VE samplers are positioned so the opening of the VE samplers are 1.37 to 1.68 m above floor level, as specified by the Cotton Dust Standard; in the cardroom the VE samplers are normally positioned 2 m above floor level because dust levels are not influenced by movement of personnel and equipment during normal test procedures.

In-house tests demonstrated no differences in dust levels at 2 m above floor level and 1.37 and 1.68 m above floor level. Each sampling station is controlled by a cut-off valve located in the vacuum line. Plastic tubes with critical flow orifices connect each sampling station to the valve in the vacuum line. The exact time of exposure for each set of dust samples can be regulated and recorded by an elapsed time meter or electrical timer located in the electrical circuitry of each vacuum line. Vacuum pressure is monitored by pressure gauges located throughout the vacuum system.

Four area samplers are located in each cardroom and each exposure chamber. The area samplers used are open-face filters that operate at 1.5 L/min. Dust measurements are determined by gravimetric methods (Hawley and Charell 1980, Perkins 1975) using 37-mm-diameter, 5-μm polyvinyl chloride filters for both the area samplers and the VE samplers. Critical flow orifices maintain the specific rates at each sampling station. These orifices are calibrated periodically with a wet test meter and fall within the limits of 7.4 ± 0.2 L/min (for the VE samplers) or 1.5 ± 0.2 L/min (for the area samplers). These orifices can maintain a constant flow rate for any pressure ratio or pressure differences in excess of one-half of an atmosphere.

A microprocessor-based electro-optical sampling instrument, usually referred to as a continuous aerosol monitor (CAM), is located adjacent to each VE sampler (Shofner et al. 1979). Each CAM sensor is connected to a control unit and printer

located in the data acquisition center inside the pilot plant structure. The CAM system is capable of electro-optically weighing the small-particle component of sampled air as mass and sizing the large-particle component according to projected area. The dust level measurements are equivalent to those made by the VE sampler (Shofner et al. 1979, Cocke and Bragg 1984) and have the advantage over gravimetric determinations in that the readings can be instantaneous or read as the latest 5-min average. The rapidity at which dust level can be determined permits adjustments of the processing variables in order to obtain the desired dust levels. Data can be stored for the duration of the particular run, then converted to mass concentration via an algorithm and printed according to the operating mode and sample time.

When quantities of dust in excess of that obtained with the VE and area samplers are required, high-volume samplers (55 ft^3/min) may be used. These samplers are not size selective, and the size of most of the dust they collect exceeds the 0 to 15 μm size range of particles collected with the VE sampler. The high-volume sampler uses standard glass-fiber filters that are 20.32 cm by 25.4 cm. In the exposure chambers, the vacuum pumps are connected remotely from the filter holder to minimize the noise levels; however, in the cardroom the vacuum pump is connected directly to the filter holder.

For biological analysis, Andersen Impactor samplers are used (Andersen 1958, Millner et al. 1985). The samplers are connected to cutoff valves in the vacuum line and are positioned so that the openings of the samplers are 1.37 to 1.68 m above floor level at each sampling station. Particles entering the sampler are deposited on plates throughout the sampler according to particle size; large particles are deposited on the top plates, and small particles on the bottom plates. Flow rate through each sampler is maintained at approximately 28 L/min with critical flow orifices.

Cotton dust can be further characterized by use of an automatic particle sizer (APS) (Cocke and Bragg 1984, Harrison and Cocke 1984b). This sampler uses a two-spot laser velocimetry technique to monitor the velocity of particles leaving an internal accelerating nozzle. The basic concept of the APS is that small particles accelerate more rapidly than larger particles; thus, the particle velocity is inversely proportional to the aerodynamic size of the particle. The instantaneous measurement of the velocity by the microcomputer system allows real time analysis of particle size, and data are available in graphical and tabular forms. The microcomputer and other system elements are located in the data acquisition center, but the APS sensor is portable and permits sampling the dust-laden air in the cardrooms and in the exposure chambers.

Dust Generation and Distribution

Since the model cardroom is part of an operating pilot plant, early studies on humans posed problems by not allowing efficient use of other plant equipment and personnel. For instance, even though the supply air passed through a high composition filter and electrostatic precipitator, no other cotton could be processed in the part of the building where the supply air originated because the agent for byssinosis was unknown. This meant that the opening and picking line and the drawing frames and roving frame operations had to be curtailed for the duration of the study.

This situation has now been alleviated by taking the supply air directly from outside the plant structure and conditioning the air before it enters the cardrooms (the capacity to use supply air from within the building still exists). This eliminates any contamination from within the plant that might affect dust composition. The supply air is cooled by passing the air through an air conditioner when the outside ambient air exceeds 32 °C or is heated by passing the supply air over a steam coil in the line when the outside ambient air is less than 4 °C. The air is then scrubbed by passing it through a wet-wash ionizing chamber and an electrostatic precipitator. The air temperature is controlled by a chill water coil and a modulated-steam heating coil. Humidity is regulated by a modulated-steam humidifying system located within the air-ducting system. The supply air then moves into the cardroom through vents that are near the ceiling and designed to minimize the effect of air currents on the dust-generating characteristics of the cards. The supply air can be varied from 0 to about 62 m^3/min by adjustment of a valve in the ducting system.

The amount of dust released by the card into the supply air in the cardroom is regulated by a separate air-handling system. The desired dust level within the cardroom is obtained by adjusting the airflow rates of dust collectors, which are located at points on the card where dust concentrations are greatest. The exhaust air from the dust-collection system passes through an air cleaner before being discharged into the air system of the main plant. The amount of air available to transport dust to the remote exposure chambers is the difference between the supply air and the air on the card dust collection system. This dust-laden air is removed from the cardroom through a venting system near the floor on the opposite side of the room from the source of the supply air. The positive air pressure drives dust-laden air through ducts to the remote exposure chambers. The air enters the exposure rooms though a "Y" duct stubbed through the wall near the ceiling. In-house tests demonstrated that no additional distribution ducts or diffusers were necessary for uniform dust distribution in the exposure chambers.

The exposure chambers are each equipped with a heat pump system that has a nominal flow rate of 1,200 ft^3/min. The heat pumps were modified and installed to operate in a vertical position. Inspection and maintenance of heat pumps is possible from within the exposure chambers. In addition to the dust-laden air, clean air enters the exposure chambers through three floor vents on each side of the chambers. The return vent is located at the end of the chamber opposite the wall where the dust-laden air enters.

Since the exposure chambers are under positive pressure, two pressure-operated vents on the opposite wall from where the dust-laden air enters are used to remove "excess" air. This excess air is ducted and discharged below the exposure chamber. A skirt and vent system around the base of the exposure chambers limits the effect of wind on the discharge of this excess air. When large quantities of dust are needed for analysis, a high-volume dust sampler located within the duct below the vent is activated.

The facilities have been downsized since the heyday of the human studies in the early 1980's. Currently, one of the exposure chambers is used for dust studies and the other is used as the control center. Only one card is dedicated to the dust studies and has been upgraded with a chute feeder (chute feeder manufactured by American Truetzschler, Inc., Charlotte, NC) so that it can be chute fed and lap fed. However, flexibility in the system design would permit reversion to large-scale dust studies as required.

Characteristics of Dust Generation and Distribution

Many idiosyncrasies were observed in dealing with the generation, measurement, and distribution of cotton dust, and procedures had to be developed to deal with them. Some of these problems may appear to be trivial, but many small and large tests were conducted to help achieve consistent results and confident interpretation of results. Some of these observations and problems resolved will be described briefly.

Work Practices and Airflow

During short sampling periods, normal work practices cause increases in dust levels. For long sampling periods, dust levels are not affected by normal work practices. Laying the lap, cleaning the scavenger roll, and cleaning under the card are the major activities that result in short-term and variable increases in dust levels. In addition, different workers performing the same task affect dust levels differently. To minimize this effect, work practices of individual workers were monitored closely, and standard operating procedures were developed. Continuous monitoring is required to determine if revisions in procedures are needed.

The effect of air currents on dust levels within the cardroom and especially in the area of the sampling instruments should be minimized. In the cardroom, for example, the incoming air is directed laterally across the room as the supply air enters near the ceiling through three sidewall diffusers with an opposed blade volume control. Air currents and diffusers can be adjusted to minimize turbulence near the sampling stations by use of smoke bombs as visual aids. Turbulence increases when the volume of supply air increases, but this occurs almost exclusively in areas near the wall and

ceiling. Air turbulence surrounding the sampling stations can be checked by releasing small particles near the stations and by observing their movement. The sidewall diffusers can be adjusted as needed.

Card production rate significantly impacts dust level; fortunately the relationship is linear. A twofold increase in the production rate results in a twofold increase in dust level. Increasing the supply airflow rate to the cardroom with no increase in the card exhaust system airflow rate reduces the dust level linearly. Increasing the card exhaust system airflow rate while keeping the supply airflow rate constant also reduces the dust level, but this relationship is exponential.

Weight of Dust on Filter

The optimum dust weight on VE filters in both the card and exposure rooms ranges from about 0.3 mg to 1.0 mg (Perkins 1975). When the quantity of dust is less than 0.3 mg, technique and procedural differences between technicians and small errors in weighing become magnified. When there is a large quantity of dust, usually anything more than 1.0 mg, particles frequently become dislodged during weighing and handling. Generally, when lint fly levels in the sampling area are high, lint fly becomes a large proportion of the sampled dust and these large particles tend to be easily dislodged. Theoretically, the design of the VE sampler precludes capture of particles in excess of 15 μm, but most filters trap some particles larger than 15 μm (Claassen 1981).

Tests have shown that some filters containing as little as 0.3 mg of dust may lose a significant amount of dust weight when the filter is grasped with forceps and subjected to rapid and vigorous movement. However, other filters with as much as 3.0 mg of dust do not lose weight when subjected to the same treatment. Apparently, factors other than weight alone determine the optimum amount of dust that should be collected on the filter.

Dust Level

Cardroom dust level is dependent on the dust potential of the cotton being processed and on the processing variables. Card production rate, air supply rate and air exhaust rates are the processing variables that have major impact. Dust level is directly related to card production rate and inversely related to the air supply and exhaust rates. To obtain a desired dust level in the cardroom, a suitable production rate or air supply and exhaust rate must be established. The processing variables are held at constant conditions when the objective is to determine differences in dust levels between test cottons. In tests that require that the dust level remains constant, either one or both processing variables are adjusted to obtain the desired dust level.

Because the amounts of dust collected on the dust-sampling filters are so small, background dust level can constitute a significant portion of the total dust level. The background dust level can be greatly influenced by an improperly designed or improperly operating air filtration system. For instance, solids could be introduced into the room atmosphere through the humidifying system (Batra et al. 1980). Depending on the humidifying system, especially in regard to the aerosol spray humidifiers found in many commercial textile mills and other model cardrooms, background levels approaching or even exceeding the Cotton Dust Standard of 200 $\mu g/m^3$ can occur. For this reason the air system must be monitored periodically to determine background dust levels in the cardroom. Also, it is imperative that dust from one cotton not be contaminated with that from another cotton in human studies. To minimize this possibility, a cleaning procedure was established to assure that the background dust level did not exceed 40 $\mu g/m^3$. Before cotton is processed for use in human studies, all machinery, equipment, fixtures, and walls and floor are thoroughly cleaned. However, less rigid cleaning procedures may be used if human studies are not involved and if the only test objective is to determine differences in dust levels between cottons. In-house studies indicated that background dust levels in the cardroom had no effect on the test results when standard cleaning procedures were used.

Selection of Processing Parameters

The processing parameters used in the card are greatly influenced by the quantity of cotton available and the dust potential of the cotton. If the amount of cotton is limited, processing conditions can be selected to extend the processing time or

elevate the dust level within the cardroom to a level that allows an adequate number of replicated dust samples to be obtained. Generally, test cottons are analyzed for dust potential and the dust potential of the "cleanest" cotton determines the card production rate for all test cottons. The airflow rates are then adjusted to produce the desired or projected cardroom dust level. The dust level in the remote exposure chamber can then be projected on the basis of cardroom dust level. To gauge the projected dust level in the exposure chamber, losses occurring in transporting the dust from the cardroom to the exposure chamber and the dilution rate for mixing dust-laden air with recirculating air in the exposure chamber have to be taken into account. The first step is to adjust the airflow rates in the cardroom supply air system and the card dust collection system to produce the desired cardroom dust level, with the supply air system flow rate approximately 650 ft^3/min in excess of the card dust collection system exhaust rate. The difference between the airflow rates of the supply air system and the card dust collection system is the amount of air available for transporting the dust to the exposure chamber.

Equilibrium should be attained before fine adjustments, dust sampling, or dust exposures are made. Determining when equilibrium conditions are met is especially difficult when dust levels are low because long run times are required to get enough dust for accurate gravimetric determinations. For this reason the CAM system is often used to determine whether equilibrium has been attained. For high dust levels, equilibrium conditions are reached in the cardroom approximately 15 min after processing is initiated. Approximately 20 min are required for the dust level in the exposure chambers to stabilize. If the dust level is below the projected level for the cleanest cotton, the card production rate can be increased or the airflow rates varied until the desired level is achieved. If the dust level exceeds the desired level, the dust level can be reduced without altering the particle size distribution or other characteristics of the dust particles. This reduction can be achieved by bleeding off a portion of the dust-laden air through a vent to the outside located in the duct between the cardroom and the remote exposure chamber.

Advantages of a Model Cardroom and Remote Exposure Chambers

The advantages of the facilities described are many. The model cardroom presents a means of generating, measuring, and controlling dust levels. The facility allows researchers to evaluate the effect on cotton dust levels from variables such as harvesting methods, gin processing machinery types and sequences, and special treatments of cotton. Unlike laboratory methods of generating and controlling dust levels, the card used in the model cardroom is a commercial card. The conditions generated in the model cardroom are identical to those found in a textile mill. But in a commercial textile mill, a researcher would have difficulty conducting an effective experiment because the variables could not be controlled properly. In addition, the ability to alter conditions in a commercial mill is drastically reduced.

The model cardroom itself can be used for animal and human studies that require constant dust levels. Even greater advantages are available when the cardroom is used in conjunction with the dust exposure chambers. In the exposure chambers, uniform dust concentration throughout the room is achieved with improved comfort for panel members, freedom from safety hazards, reduced noise levels, and absence of large quantities of lint fly. With the separation of the dust generation and exposure locations, human panelists are less likely to be biased to a test cotton. Panelists can be tested for lung function without leaving the model cardroom, since the equipment, instrumentation, and supporting systems are present in the room for physical, chemical, and biological tests.

Model Cardroom Applications

The model cardroom and its adjunct exposure chambers are tools. As a tool, the use of the model cardroom can be expanded by the imagination of the researcher. Some representative uses of the model cardroom are presented in the text that follows only as examples.

Sampling of Dust and Its Associated Microorganisms

One invaluable use of model cardrooms has been to validate and compare dust measuring techniques and equipment (Cocke and Bragg 1984, Harrison and Cocke 1984b, Miller et al. 1984, Sasser et al. 1986, Millner et al. 1988). While the VE sampler is the recommended dust sampler, provisions in the Cotton Dust Standard permit monitoring with devices other than the VE sampler if the device is in agreement with the VE sampler. A good way to show correlation is by concurrent testing in a cardroom. Related to this, Fornes and Gilbert (1984) were able to collect large quantities of dust from different bales of cotton. They found that the dust separated from their model cardroom condenser trash was <20 μm in diameter and chemically similar to dusts collected in the same environment by a VE sampler. Even more extensive characterization was done by Muller and Jacks (1982) who documented a preponderance of unidentifiable particles and plant fragments and ubiquitous presence of bacteria and fungi from dust samples collected from plates of the electrostatic precipitator in the exhaust air line in the Clemson model cardroom.

Welty et al. (1977) first used the Andersen air sampler to characterize the microbial population found on cotton dust. Since then, by the use of selective media and replica-plating procedures, the bacterial composition of cotton dust has been examined extensively (Millner et al. 1983, 1985; Chun 1989, 1990). Now, cottons grown in different years and locations can be characterized and categorized according to populations of bacterial types in the generated dust. A word of caution should be interjected here about interpreting these results because (Chun and Perkins 1991) gram-negative bacteria have been shown to decrease in proportion to the total bacterial population during storage of lint. This is an important fact because gram-negative bacteria are associated with endotoxin and endotoxin is associated with byssinosis (Schneiter et al. 1942, Cinkotai et al. 1977, Helander and Lounatmaa 1981).

Dust Reduction by Washing Cotton or Using Additives

The model cardroom enables researchers to study cotton dust characteristics and control of causative agents of byssinosis, and the information gained from this research will eventually lead to a complete solution to the cotton dust and byssinosis problem.

Perhaps one of the most important correlations established from research in model cardrooms is that between airborne endotoxin concentrations and decreases in human pulmonary functions (Castellan et al. 1984, Rylander et al. 1985, Castellan et al. 1987). This finding has led to many attempts to reduce not only the dust content but also the amount of endotoxin associated with cotton. Boehlecke et al. (1981) found that the adverse human pulmonary response to dusts generated by cottons obtained from aseptically harvested closed bolls was much less severe than the response to dusts from the corresponding normally harvested cottons. However, the use of cotton harvested from green, closed bolls is not commercially feasible (Bargeron et al. 1981, Cocke et al. 1981). Perhaps the most promising approach for purifying cotton follows along the lines originally suggested by Merchant et al. (1973) who attempted to clean or deactivate the toxic material in cotton lint. This approach has been carried out further by washing cotton to successfully reduce both the potential for dust generation and endotoxin levels while still retaining good processing qualities (Perkins 1981; Ross et al. 1981; Perkins and Cocke 1982; Cocke et al. 1983; Millner et al. 1983; Olenchock et al. 1983; Sasser and Perkins 1984; Olenchock et al. 1986; Petsonk et al. 1986; Wakelyn 1986; Perkins and Berni 1990, 1991).

Another finding from research in the model cardroom is the effect of additives on dust level. Application of certain additives to cotton has been shown to reduce dust levels and bring about changes in the dust characteristics (Carter et al. 1980; Cocke 1981b; Perkins and Cocke 1988). When oily additives were used in two textile mills, the levels of dust and associated levels of bacteria and endotoxin were reduced significantly (Fischer et al. 1984).

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Chapter 15. Effects Induced by Microbial Products Found in Cotton Dust

Ragnar Rylander

A broad range of species of microorganisms are found on cotton and in cotton dust, including gram-negative and gram-positive bacteria as well as molds. In comparison to other organic dusts, dust from cotton is unique because it generally contains less mold. Excessive mold growth on cotton after harvest results in a product of inferior quality, and this cotton is usually rejected before ginning. Thus the ratio of bacteria to molds in cotton dust is quite different from the ratio for other organic dusts, for example, moldy hay or waste products such as compost.

The microorganisms present on cotton contain a large number of substances having potential biological activity in humans, but only a few have been extensively investigated. A need exists for further research to evaluate which and how these specific substances cause inflammatory responses in the airways and other reactions that occur after the substances are inhaled from cotton dust (Rylander et al. 1987). Even if a relationship has been found between a particular substance and an effect among workers exposed to cotton dust, this does not mean that that substance is in fact the cause; instead the substance could just be a marker of other agents present in the dust that are the real causative agents.

Reactions After Cotton Dust Exposure

Biologically active agents initiate reactions through activation of cells. What we observe as clinical symptoms or disease is the end result of a multitude of reactions, often involving a whole series of cell systems working together. Examples are the activation of macrophages and the subsequent secretion of cytokines (proteins with particular activities), which cause other cells, notably neutrophils and eosinophils, to migrate into the tissue. Cytokines also activate T-cells, and, after a renewed exposure, these cells can then react directly to the stimulation (Burrell 1990). Knowledge of the mechanisms behind inflammation is necessary to understand the pathogenesis behind cotton dust induced effects (Henson and Murphy 1989). The information obtained from isolated in vitro cell systems is very limited, and it is difficult to explain disease from these systems. It is particularly important to realize that a negative outcome of an exposure of a single cell to an agent does not rule out that it may be active in vivo.

Experimental and epidemiological studies on biological agents in cotton dust have an ultimate goal of defining causative agents so that suitable and effective prevention can be undertaken. Conclusions on causality in human and animal studies must be based on the following criteria:

Animals—relevant exposure, route and dose, relevant models, effects related to clinical disease;

Humans—dose-response relationships, reactions at appropriate dose levels, challenge experiments with purified substance.

In this list, challenge experiments with the pure agent are particularly important because they provide critical information on causality according to well-established epidemiological criteria.

Experiments that expose human subjects to cotton dust for long periods are not possible for ethical reasons. Several reactions occur after an acute exposure, such as Monday morning chest tightness and decreases in FEV_1 during the workshift, and these reactions can be studied in cotton workers or persons not previously exposed to cotton dust (naive subjects). Certain reactions after cotton dust exposure, particularly chest tightness, require repeated exposures before they occur, which suggests that a mechanism of sensitization exists. For such effects, experiments on subjects not previously exposed will not give the relevant information.

Experience on specific microbial products in cotton dust and the reactions after exposure is only available for endotoxin from gram-negative bacteria (GNB), ß-1,3 glucan from molds, proteases mainly from gram-positive bacteria, and mycotoxins from fungi. In the text that follows, each of these agents will be reviewed with respect to their nature, origin, and ability to induce an inflammatory response or disease.

Endotoxins

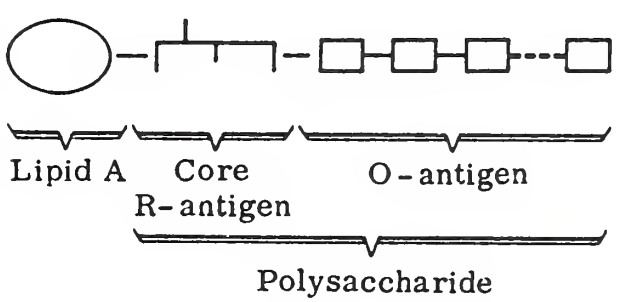
Sources and Characteristics

Endotoxins are a constituent of the external wall of GNB, microorganisms that are always present on cotton plants. Endotoxins are made up of a complex lipopolysaccharide (LPS) compound (illustrated in fig. 11). An LPS consists of polysaccharide chains connected by a core polysaccharide KDO (3-deoxy-D-manno-2-octulosonic acid) covalently bound to a lipid part (lipid A). Almost always the lipid part consists of a series of long-chain fatty acids connected by amide and ester linkages to a diglucosamine structure.

The lipid A part causes the formation of micellae in water suspensions and is responsible for the majority of the toxic properties of endotoxin. The polysaccharide part accounts for the serological specificity of LPS and determines the solubility of the molecule in water.

Although the terms endotoxin and LPS are often used interchangeably, the two expressions are not synonymous. Endotoxin refers to the toxin as present on the bacterial cell wall or fragments thereof. Thus the exposure to endotoxin under real conditions also includes bacterial proteins and other cell constituents. LPS implies a chemically purified endotoxin with no or only trace amounts of cell wall protein. This preparation, obtained by extraction with a phenol-chloroform-water mixture, is an artificial preparation that is not present in cotton dust. In aerosols of water contaminated with GNB, however, free macromolecular aggregates of LPS may be present.

Figure 11. Schematic structure of lipopolysaccharide



Biological Effects

The general toxicity of endotoxin has been studied extensively (Burrell 1990), and reviews of the effects of endotoxin on the lung have been published (Brigham and Meyrick 1986). Most of the previous work and all of the work cited in that review, however, dealt with endotoxin administered by injection. The effects of the inhalation of endotoxin must be considered separately, since the responses, in the lung and in the blood, are different from the effects observed after administration by injection. The explanation that follows deals exclusively with the effects of endotoxin after inhalation.

The chief cell in the lung responsible for the defense against external agents is the macrophage. These cells are present in the airways (airway or alveolar macrophages) and in the lung wall. The latter type is more reactive to environmental challenges and determines the initial reactions after inhalation.

The alveolar macrophage is the primary target for endotoxin after inhalation. Very little of inhaled endotoxin remains free or penetrates through the lung tissue into the blood (Goto and Rylander 1987). Endotoxin is chiefly taken up by pinocytosis when it occurs in a macromolecular form (LPS) and by phagocytosis when attached to dust particles.

Endotoxin has extensive effects on the inflammatory and immune cells of the body (Burrell 1990). It induces a series of intracellular changes in the macrophage that can be collectively referred to as activation. After endotoxin challenges the macrophages, these cells respond to a standard stimulus by producing free oxygen radicals. This increased state of reactivity persists up to 48 hr after exposure. The activation of macrophages also results in the production of chemotactic factors, particularly neutrophil chemotactic factor. The presence of neutrophils newly recruited from the blood characterizes the acute cellular response to inhaled endotoxin (and to cotton dust) (Venaille et al. 1989). This invasion by neutrophils takes place in the lung wall very shortly after exposure and peaks a few hours thereafter; a subsequent invasion occurs in the airways and peaks after 24 to 48 hr.

Endotoxin-induced activation of macrophages also results in an increased production of lysosomal

enzymes as well as the production of different cytokines, such as platelet-activating factor (PAF), interleukin-1 (IL–1), and tumor necrosis factor α (TNF–α) (de Rochemonteix et al. 1991). It is generally believed that cytokines are important mediators for later inflammatory events (Morrison and Ryan 1987). Production is initiated almost immediately after exposure, and peak production occurs 1 to 2 hr thereafter. Although an initial exposure to endotoxin causes the production of TNF–α and PAF, subsequent exposures to endotoxin cause a tachyphylactic response in the production of these two cytokines (that is, subsequent exposures do not cause production of these cytokines). However, in the case of IL–1, a second exposure causes a renewed production and the amount produced is even greater than that produced after the first exposure.

TNF–α stimulates T-cells to increase the expression of major histocompatibility antigens and IL–2 receptors and affects hematopoietic stem cells in various ways. An injection of purified TNF–α causes fever. Many of the endotoxin-induced effects can be attributed to the production of TNF–α , although some, such as septic shock, cannot be mediated by TNF–α alone. Endotoxin also stimulates endothelial cells, which induce procoagulant activity and produce several inflammatory mediators (Burrell 1990).

Reactions occurring after repeated exposures are most important for the understanding of events following inhalation of cotton dust containing endotoxin. It has already been mentioned that an initial exposure to endotoxin alters the reaction pattern to a subsequent exposure with regard to the cytokines TNF–α and IL–1. Evidence from animal experiments demonstrates that the acute neutrophil invasion into the lung tissue is diminished after repeated exposure to endotoxin and that the predominant cell in the airway tissue after repeat exposures is the eosinophil. Data from animal experiments also suggest a repeated exposure to endotoxin causes platelets and probably also blood monocytes to become sensitized. A smaller dose of endotoxin is required for platelets to adhere to one another and to activate blood monocytes to produce inflammatory mediators, such as procoagulant factor.

Relation to Symptoms Induced by Cotton Dust

There is now little doubt that acute episodes of fever, chills, and malaise (toxic pneumonitis), which have sometimes been called "mill fever," are caused by bacterial endotoxins. The cellular mechanism responsible for this disease is probably the secretion of TNF–α from activated macrophages in the lung. Twenty-four hours after exposure, TNF–α has been demonstrated in the blood of subjects who inhaled endotoxin (Michel et al. 1989). Repeated exposures to endotoxin cause the development of tolerance, and fever is no longer present. This can be explained by tachyfylaxis of the secretion of TNF–α and other inflammatory mediators. However, even if tolerance to fever develops, other reactions induced by endotoxin remain.

In cotton workers, an increase in breathing frequency after carbon dioxide stimulation was originally described by Prausnitz (1936). This effect has also been demonstrated after the inhalation of endotoxin (Rylander et al. 1989a) and probably reflects the development of a toxic pneumonitis with an increased receptor sensitivity in the alveolar walls. Similar observations have been made in animal models.

Inhaled endotoxin also causes a dose-related bronchoconstriction that develops 4–6 hr after exposure (Michel et al. 1989, Rylander et al. 1989a). The response can be measured with spirometry by determining the forced expiratory volume in 1 sec (FEV_1). The same effect is found after exposure to cotton dust containing endotoxin (Haglind and Rylander 1984, Rylander et al. 1985). The effect is more accentuated among persons with reactive airways (Michel et al. 1989). Chapter 16 in this book reports on the dose-response relationship between endotoxin and chest tightness (Cinkotai et al. 1977), and endotoxin and the overshift decrease in FEV_1 demonstrated among cotton workers (Rylander et al. 1985, Rylander and Haglind 1986), naive subjects (Haglind and Rylander 1984) and naive subjects with sensitive airways (Castellan et al. 1984).

Inhalation of endotoxin can also increase the reactivity of the airways. Increased reactivity is more likely in persons with previously high reactivity and particularly in persons with clinical asthma

(Haglind and Rylander 1984). The limited data available also suggest that the airway responsiveness is increased among cotton workers in comparison to unexposed controls and that it increases over the workshift.

Inhaled endotoxin may induce a subjective feeling of chest tightness (Schilling et al. 1955). This symptom, which is a classic symptom of byssinosis, occurs on Mondays or other days when the workers return to work after an absence. In modern cotton mills with low dust levels, the symptom may appear on other days when there is an unusually high exposure to cotton dust, for example, when machines are cleaned. The symptom is generally found in cotton workers exposed for several years. Experience from experiments in cardrooms has demonstrated that the symptom can be produced among previously unexposed subjects, but only at high dose levels of cotton dust. This suggests that the underlying mechanism for chest tightness among cotton workers is related to sensitization, presumably of platelets.

Chronic inflammation develops in the airways after exposure to airborne endotoxin over longer time periods. The development of goblet cell hyperplasia and an increase in mucus secretion have been demonstrated in animal models (Pernis et al. 1961, Snell 1966). These histological observations are similar to those made in studies on nonsmoking workers in cotton mills (Pratt et al. 1980).

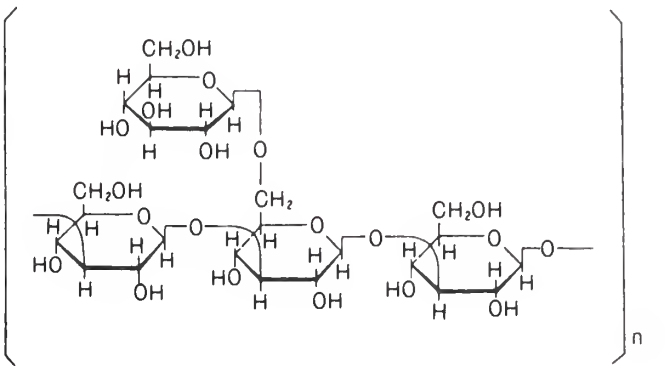
After a person has been chronically exposed to cotton dust, the number of eosinophils in the subepithelial layer of the airway mucosa increases. Endotoxin also has the potential to boost responses to inhaled antigens, and these phenomena could be related to the symptoms of wheezing that occur among certain cotton workers and the increased reactivity of the airways. In comparison with the acute effects, the effects after long–term exposure to endotoxin are less well studied, however, and the few data available must be interpreted with care.

Beta-1,3 Glucans

Sources and Chacteristics

Glucans are compounds that are widespread in plants and microorganisms, particularly in the cell

Figure 12. Schematic structure of (3→1)-ß-D-glucan



structure of molds. Basically, glucans are polyglucose compounds in which glucopyranosyl rings are attached in chains and sometimes with side chains. The chains may be linked in α or ß positions with 1–3, 1–4, or 1–6 linkages. Glucans with ß–1,3 linkages have potent biological effects. Figure 12 illustrates the structure of a ß–1,3 glucan.

In addition to the linkages between chains, glucans are also characterized by the structure of the polyglucose chain. This chain may be straight with side chains or may form helices (mainly triple helices). Glucans that are gently extracted—for example, schizophyllan from mushrooms—are present in a triple helix configuration. A triple helix configuration can be transformed to a random coil by heat treatment (at 135 °C) or alkaline treatment (using 0.10–0.15 N NaOH).

A major source of ß–1,3 glucans is the cell walls of fungi. They are also produced by *Actinomyces/Streptomyces*, *Alcaligenes faecalis*, and certain mushrooms. Only a limited amount of data is available on ß–1,3 glucans in cotton dust. Quantities of up to 1 μg/m^3 have been measured in the air of an experimental cardroom (Rylander et al. 1989b). It is likely that the levels are even higher in environments where materials such as moldy hay are handled.

Biological Effects

Many publications have reported the effects of ß–1,3 glucan on different cell systems. A review of these publications was made by Di Luzio (1985). The major pathway through which ß–1,3 glucans

exert their activity is through phagocytosis by macrophages (Benacerraf and Sebestyn 1957, Riggs and Di Luzio 1961). The macrophages have specific glucan receptors on their surface.

A major consequence of macrophage stimulation is the secretion of colony-stimulating factor (CSF). CSF influences the various parts of the reticuloendothelial system (RES), causing the secretion of Il–1 and affecting T4 (helper) cells (Di Luzio 1985).

There is agreement that ß–1,3 glucans from different sources may have different biological effects. Whether this is determined by the molecular configuration or by other factors is not known. With regard to the biological activity of different configurations, data are still controversial. Some observations suggest that the triple helix is an active antitumor configuration, while other researchers propose that the random coil configuration is more active.

Of particular interest is the capacity of ß–1,3 glucan to prime different cell systems. In comparison to bacterial endotoxin, ß–1,3 glucan is a potentially weaker primer for cells, for example, for secretion of TNF–α . However, the duration of the priming is longer—about 16 hr for glucan as compared with 8 hr for endotoxin.

In most of the studies published, ß–1,3 glucan has been administered by injection into the blood or the peritoneum or the effects have been evaluated using in vitro methods. Some data are available on the effects after inhalation (Fogelmark et al. 1992). The results from a study on guinea pigs suggest that inhalation of glucan causes an inflammatory cell response that is basically different from the response from inhalation of endotoxin. At 4 and 24 hr after exposure, no invasion of neutrophils into the lung wall or the airways was detected. However, a decreased number of macrophages and lymphocytes were found in the airways and lung walls. This decrease persisted up to 7 days after a single exposure. Such prolonged effects have also been seen in studies in which ß–1,3 glucan was administered by injection (Di Luzio 1985).

At present no data exist that show the effects of inhalation of airborne ß–1,3 glucan in cotton dust. Since ß–1,3 glucans are potent stimulators of T4 cells, it can be hypothesized that they, by themselves or by interacting with endotoxin, play a role in the development of airway inflammation and allergic asthma that occurs in some persons exposed to cotton dust.

Proteases

Sources and Characteristics

Proteases are enzymes that cleave peptides. Proteases are produced by gram-positive bacteria and have been commercially used as detergents. Gram-positive bacteria are common in cotton dust. Airborne levels of proteases have been measured in cotton mills (0.15–1.2 μg/m^3 in cardroom air). In a willowing mill that handles waste cotton, the concentration was 10.7 μg/m^3 (Chinn et al. 1976, Cinkotai 1976).

Biological Effects

Proteases can destroy proteins and peptide conjugates within the cells and the extracellular matrix. Proteases can induce vascular permeability in rat skin and induce non-IgE-mediated mast cell degranulation. Exposure to purified proteases can cause hypersensitivity and occupational asthma. They are also thought to be responsible for the sensitization to the house dust mite because they are present in the feces of the mite. Proteases could theoretically induce an activation of macrophages and T-cells, leading to sensitization. No data to support this hypothesis are available from experiments with animals with controlled exposures in levels equivalent to those present in cotton mills.

Relation to Effects Induced by Cotton Dust

In studies in the United Kingdom, no relation was found between the exposure to airborne proteases and chest tightness. There were, however, indications that exposure to proteases was related to symptoms of chronic bronchitis (Chinn et al. 1976, Cinkotai 1976). Proteases are sensitizing agents and could be related to the cases of occupational asthma that develop in cotton mills.

Mycotoxins

Sources and Characteristics

Mycotoxins are a group of relatively low molecular weight metabolites of the filamentous fungi. A direct relationship usually exists between the presence of mycotoxins and the occurrence of mold in material. Although the mold in cotton may no longer be viable after processing and storage, mycotoxins remain intact.

The mycotoxins are characterized by a diversity of chemical structures. The genera *Aspergillus, Penicillium,* and *Fusarium* are the most important potential producers of mycotoxins, and each contains a number of toxigenic species of considerable importance (Moss 1989).

In occupational environments, the effects of exposure to mycotoxins in grain silos have been evaluated, and aflatoxin B1 has been reported in lung tissue of persons with lung cancer associated with aspergillosis. No data are available on the effects of mycotoxins in cotton dust.

The effects of mycotoxins are difficult to demonstrate, and the analytical methods involved in doing so are complicated. No routine measurement techniques are available, for instance, to analyze a large number of filters from a cotton plant.

Biological Effects

The aflatoxins produced by *Aspergillus flavus* still dominate the research efforts on mycotoxins. The acute and chronic toxicity of aflatoxins, including carcinogenicity, are well documented for a diverse range of animal species, and the molecular and cellular bases of this toxicity are relatively well understood. Aflatoxins are acutely toxic, carcinogenic, and immunosuppressive. Particularly active compounds develop after the aflatoxin is metabolized in the liver.

Another type of mycotoxin is tremorgenic mycotoxins derived from *Aspergillus, Penicillium,* and *Claviceps*. These mycotoxins are probably metabolized in the liver like aflatoxin is. Tremorgenic mycotoxins affect the central nervous system, a possible mode of action being interference with cerebrocortical nerve endings, causing changes in the amino acid neuron transmitter release mechanisms. These effects on the nervous system occur even from low levels of tremorgenic mycotoxins (on the order of a few micrograms per kilogram body weight).

The gliotoxins are secondary fungal metabolites with a wide variety of effects. These toxins are immunosuppressive through binding the receptors on the surface of T cells, thus inhibiting their normal function. Gliotoxins trigger natural killer cells and may activate nuclease enzymes, inducing fragmentation of cell DNA.

Relation to Effects Induced by Cotton Dust

No studies have been performed on humans to show the effects of mycotoxins inhaled from cotton dust. The risk for such effects is probably less after cotton dust exposure than after exposure to other organic dusts, particularly those from moldy materials. First of all, as indicated earlier, cotton is generally selected for processing only if it is low in mold contamination. Second, the typical symptoms seen after exposure to cotton dust in terms of an acute or an inflammatory response, sometimes accompanied by classic occupational asthma, seem to be consistent and do not mimic the symptoms of mycotoxin exposure. For example, symptoms of mycotoxin exposure—excessive fatigue, malaise, headache, or neurological symptoms—have not been observed in other experiments evaluating the effects of cotton dust. However, occasionally such symptoms are found among farmers and other workers exposed to moldy dust.

The possibility of mycotoxin interacting with other agents that cause symptoms when cotton dust is inhaled (such as endotoxins) cannot be ruled out. From a toxicological point of view, however, such a synergism seems less likely since the prime site of action for endotoxins and ß–1,3 glucans is macrophages in the lung, whereas the primary site of action for mycotoxins is in the liver, the bone marrow, or the central nervous system.

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Chapter 16. Endotoxin as an Etiologic Agent of Byssinosis: Evidence From Experimental and Epidemiological Studies Involving Human Exposure to Cotton Dust

Robert M. Castellan

During the decade and a half since the promulgation of the OSHA Cotton Dust Standard (OSHA 1978), results of new studies have intensified interest in the hypothesis that inhalation of gram-negative bacterial endotoxin causes byssinosis. This chapter reviews historical and recent clinical and epidemiological research relevant to endotoxin as a possible causative agent of byssinosis.

Historical Findings (Through 1970)

Ramazzini (1713), describing the use of animal dung as an agent used to facilitate retting of flax and hemp, recorded what may be the first observation of direct relevance to the possible etiologic role of endotoxin in byssinosis: ". . . hemp and flax are macerated in stagnant, putrid waters and are first smeared with filth to hasten the necessary maceration when they are submerged under water, and so the particles that the carders breathe in must be poisonous and highly injurious to human beings."

The first clear suggestion that endotoxin may cause respiratory disease associated with the inhalation of cotton dust came as a consequence of a U.S. Public Health Service investigation of an outbreak of acute respiratory illness among individuals making mattresses from a stained low-grade cotton. The severity of illness varied with degree of exposure, and the symptoms started 1 to 6 hr after work with the cotton began and lasted 2 to 5 days. Along with fever, fatigue, generalized aches, anorexia, headache, nausea, and vomiting, many affected individuals experienced "substernal discomfort or pressure, varying in intensity and duration, so that the person was unable to take a deep breath" (Neal et al. 1942).

The cotton associated with these outbreaks was extremely dusty and heavily contaminated by a gram-negative organism referred to by Neal et al. (1942) as the "cotton bacterium." This organism was found in abundance in samples of cotton from a commercial mill in which workers frequently experienced mill fever and was found in varying concentrations on other cotton samples but was not found on samples of high-grade, white cotton. Based on this evidence and results of experimental human inhalation challenges involving samples from the outbreak and other cottons, the authors concluded that the gram-negative cotton bacterium or its product(s) caused the outbreaks of mattress-maker's illness. Furthermore, they hypothesized that this gram-negative bacterium probably causes mill fever and may also be involved in the etiology of chronic lung disease among cotton mill workers.

Airborne Bacteria in Cotton Textile Mills

The presence of large numbers of gram-negative bacteria (GNB) in cotton dust had been reported earlier (Report of the Departmental Committee 1932). However, since cotton workers suffering from byssinosis were not clinically infected, this observation received little attention at the time. Later, Furness and Maitland (1952) studied the microflora in cotton dust and found in excess of 1 billion viable colony-forming units (cfu) of GNB per gram of dust. In a study reported by Drummond and Hamlin (1952) and Hamlin (1952), much higher airborne concentrations of viable bacteria were found in the cardroom than in mill areas associated with later processing stages, and airborne bacteria concentrations were also found to vary by growing region and crop year.

Tuffnell (1960a) observed a predominance of gram-positive *Bacillus* species in the air of cotton and jute mills and an apparent crude association between byssinosis prevalence and the concentration of live *B. pumilis* and *B. subtilis* organisms. A single medium was used to culture both GNB and gram-positive bacteria instead of separate selective media for each of these types of bacteria. Notwithstanding limitations of his preliminary epidemiological approach, Tuffnell (1960b) attempted to experimentally induce typical symptoms in byssinotic patients with aerosolized *Bacillus* organisms. These attempts were unsuccessful.

Roach and Schilling (1960) epidemiologically demonstrated that byssinosis prevalence correlated

with the "protein" fraction of cotton dust rather than with the "cellulose" or "mineral" fractions. Citing Tuffnell's work to discount bacterial involvement in the etiology of byssinosis, they concluded that their own finding "points to some constituent of the plant debris as the active agent." This conclusion ignored the fact that Tuffnell's work focused on gram-positive bacteria and may have served to divert research attention away from the microbiological etiology of byssinosis and toward a plant component (for example, bract) etiology.

Biologically Retted Versus Chemically Retted Flax and Hemp

Bouhuys et al. (1963) noted the absence of Monday chest symptoms and leukocytosis and the absence of a decline in acute forced expiratory volume in one second (FEV_1) among workers at a plant that processed chemically retted flax, an observation that contrasted sharply with findings of a similar survey at a plant that processed biologically retted flax. This observation suggested that the microbes responsible for biological retting may be involved in the etiology of byssinosis. Others have similarly noted that dust from chemically retted hemp is better tolerated by workers than dust from biologically retted hemp (Barbero and Flores 1967) and that dust from chemically retted flax fails to cause the significant acute decline in FEV_1 seen with dust from biologically retted flax (British Occupational Hygiene Society 1980).

The Milan Group: Epidemiological and Experimental Evidence

Pernis et al. (1961) confirmed the presence of endotoxin in cotton dust and performed a variety of animal and human exposure studies that supported endotoxin's causal role. They also briefly described an investigation of a typhoid vaccine factory where substantial concentrations of airborne GNB occurred several times a year during the centrifugation of large quantities of *Salmonella typhi*. Many workers regularly experienced transient low-grade fever following this particular process, and some were excluded from this work area because of a tendency to develop acute respiratory difficulties.

Since various taxonomically different plants (for example, cotton, flax, and hemp) had been implicated in byssinosis and related disorders, Pernis et al. (1961) concluded that a component of the cotton plant was an unlikely candidate for etiologic agent. Acknowledging the likely presence of other cotton dust components that demonstrate activity in vitro, they concluded that gram-negative bacterial endotoxin accounted for a substantial portion of the pharmacologic activities of cotton dust. Furthermore, they suggested that endotoxin tolerance (known to occur after sequential exposures) and the body's loss of this temporary state of resistance to the effects of endotoxin (upon interruption of sequential exposures) might account for the classical reappearance of byssinosis symptoms each Monday. Pernis et al. (1961) clearly favored endotoxin as the cause of mill fever, and suggested that byssinosis may be due to repeated inhalation of smaller amounts of endotoxin.

Later, this same group of researchers published what appears to be the first epidemiological evidence suggesting that the prevalence of byssinosis depends more on airborne endotoxin concentration than on airborne dust concentration (Cavagna et al. 1969). Correlations calculated from results in that report indicated that the prevalence of byssinosis was strongly correlated with airborne endotoxin concentration (r=0.99; p<0.01) but not significantly correlated with airborne dust concentration (r=0.88; p>0.12). Along with results of experimental exposures of animals and humans to *E. coli* lipopolysaccharide (LPS), this evidence provided additional support for endotoxin's role in the etiology of byssinosis (Cavagna et al. 1969). However, the authors concluded that "we are not unaware...that cotton extracts contain other pharmacologically active substances. Therefore, we are far from saying that endotoxins are the sole cause of byssinosis, but they may well be one of the causes."

Recent Experimental Studies Involving Human Exposure to Cotton Dust

For obvious ethical reasons, attempts to elucidate the etiology and pathogenesis of byssinosis by experimentally exposing human volunteers have been limited to the investigation of short-term, reversible effects of dust inhalation. Experimental human exposures that involve conditions typical of the mill industry (for example, exposure to dust

levels typically generated by a card for a period of several hours), particularly exposures to airborne endotoxin, are reviewed in the following paragraphs. Table 67 summarizes these studies. (Experimental studies of human response to inhalation of specific components of cotton dust, including LPS or GNB aerosols, are reviewed elsewhere in this book.)

Exposures to Dust from Washed Cotton

In one type of experiment using human subjects, both endotoxin contamination and potency of card-generated cotton dust (in terms of its ability to induce chest tightness and fever and decrease across-shift FEV_1) have been markedly reduced or eliminated by washing the cotton before carding (Rylander and Haglind 1981, Haglind and Rylander 1984, Castellan 1986, Petsonk et al. 1986). Although prewashing markedly reduces endotoxin content of cotton dust, the concentrations of other components are also reduced. Therefore, results of washing experiments do not by themselves represent strong support of the hypothesis that endotoxin is a causal factor of byssinosis and will not be further discussed.

Exposures to Dust from Untreated Cotton

A second type of experiment involving assessment of human responses to card-generated dust has taken advantage of natural variation in endotoxin contamination of cotton dust. Cottons of different grades and possibly from different growing regions are carded separately. These cottons vary in degree of endotoxin contamination and can therefore be used to produce different concentrations of airborne endotoxin independent of dust concentration and presumably some (but admittedly perhaps not all) other components of the dust. Several such studies have documented an association of fever and symptoms with high endotoxin concentration and an exposure-effect relationship between endotoxin or GNB and a decrease in across-shift FEV_1 (Millner et al. 1983, Olenchock et al. 1983, Rylander and Haglind 1983, Castellan et al. 1984, Rylander et al. 1985, Rylander and Haglind 1986, Castellan et al. 1987). Table 67 summarizes the results of these experimental human exposure studies.

The most clear-cut finding from a study of this type was reported by Castellan et al. (1987). These investigators analyzed results of a series of 108 separate experimental exposures (of groups of 24–35 subjects at a time) to controlled levels of dust generated from carding 32 different cottons. Airborne dust concentration did not correlate with airborne endotoxin concentration (r=0.07, p=0.46). The group mean FEV_1 response did not correlate with dust concentration (r=-0.08, p=0.43). In contrast, a clear exposure-response relationship was observed between group mean FEV_1 response and endotoxin concentration (r=-0.74, p<0.0001).

Logarithmic transformation of endotoxin concentrations clarified the relationship at lower endotoxin levels and resulted in a strengthened correlation between endotoxin concentration and FEV_1 response (r=-0.85, p<0.0001). Whereas all 51 endotoxin exposures above 50 ng/m^3 resulted in significant mean FEV_1 responses, none of the eight endotoxin exposures below 10 ng/m^3 resulted in a significant response, and a linear regression model based on the data predicted a threshold for response at 9 ng/m^3 (Castellan et al. 1987). Although these experimental results do not by themselves prove that endotoxin is causal, they strongly support the hypothesis that endotoxin plays a major role in the cause. In contrast to the washing experiments, concentrations of cotton dust components were not artificially altered in these studies.

The relationship observed by Castellan et al. (1987) between airborne endotoxin concentration and acute decline in FEV_1 is only likely to have been observed if the causal agent is endotoxin or some other cotton dust component with a concentration that closely parallels that of endotoxin. Although the concentrations of many cotton plant products do not parallel the concentration of endotoxin (Bell 1986, Bell et al. 1986), it has been suggested that concentrations of certain phytoalexins (for example, cadelenes and lacinilenes), which are toxic compounds produced by the cotton plant in response to microbial infections, might roughly correlate with endotoxin concentration (Greenblatt and Bell 1986). However, unlike the situation with endotoxin, there is no existing clinical or epidemiological evidence to indicate that these phytoalexins play a causative role.

Table 67. Results of studies in which humans were experimentally exposed to card-generated dust from untreated cotton (limited to studies in which the effects of airborne endotoxin or airborne GNB were evaluated)

Study	Maximal airborne concentrations* Dust (mg/m³)	Endotoxin (ng/m³)	GNB (cfu/m³)	Number of subjects†	Results of study
Millner et al. (1983)	Not reported (VE)	Not tested	22,900 (Andersen; gram-negative selection agar)	56 (Healthy cotton dust "reactors")	Individual FEV_1 change correlated with GNB (r=-0.51) and total bacteria (r=-0.47) (both p<0.001) and dust (r=-0.26, p<0.01) but not with fungi (r=-0.31). Six-hour exposures. Data represent a subset of data analyzed in Castellan et al. (1984).
Olenchock et al. (1983)	Not reported (VE)	Not reported (VE dust; spectrophotometric LAL)	Not tested	56 (Healthy cotton dust "reactors")	Individual FEV_1 change correlated more strongly with endotoxin (r=-0.51, p<0.001) than with dust (r=-0.26, p<0.01). Six-hour exposures. Data represent a subset of data analyzed in Castellan et al. (1984).
Rylander and Haglind (1983)	2.58 (VE)	8,030 (VE dust; gel titration LAL)	Not tested	11 (Smoking cotton workers and workers with byssinosis)	Mean FEV_1 change correlated highly with endotoxin (r=-0.96, p<0.001) but not with dust (r=-0.25). No correlation between dust and endotoxin (r=+0.06). "Threshold" at 1,000 ng/m³. Four-hour exposures. Data represent a subset of data analyzed in Rylander et al. (1995).
Castellan et al. (1984)	0.55 (VE)	700 (VE dust; spectrophotometric LAL)	Not reported (Andersen; gram-negative selection agar)	54 (Healthy cotton dust "reactors")	Mean FEV_1 change correlated with endotoxin (r=-0.94), GNB (r=-0.91), total bacteria (r=-0.71) (all p<0.00001) and dust (r=-.34, p<0.05) but not with fungi (r=-0.14). Fever occurred after highest endotoxin exposure. Six-hour exposures. Data represent a subset of data analyzed in Castellan et al. (1987).
Rylander et al. (1985)	3.16 (VE)	8,030 (VE dust; gel titration LAL)	Not tested	15 (Smoking cotton workers and workers with byssinosis)	Mean FEV_1 change correlated with endotoxin (r=-0.56, p<0.05) but not with dust (r=0.01). "Threshold" at approximately 33 ng/m³ endotoxin. No correlation between dust and endotoxin. "Mill fever" occurred after highest endotoxin exposures. Four-hour exposures.
Rylander and Haglind (1986)	3.6 (VE)	8,000 (VE dust; gel titration LAL)	Not tested	13 (cotton workers with and without byssinosis)	Individual and group mean FEV_1 change both correlated with logarithm endotoxin (p<0.01) and not with logarithm dust. No correlation between dust and endotoxin. "Mill fever" occurred after highest endotoxin exposures. Four-hour exposures.
Castellan et al. (1987)	0.55 (VE)	779 (VE dust; spectrophotometric LAL)	Not tested	33–61 (Healthy cotton dust "reactors")	Mean FEV_1 change correlated with endotoxin (r=0.74) and log endotoxin (r=0.85) (both <0.0001) but not with dust (r=0.08). No correlation between dust and endotoxin. "Threshold" at approximately 9 ng/m³ endo-in. Six-hour exposures.

*VE, Vertical elutriator.

†Cotton dust "reactors" are individuals selected on the basis of their reaction to cotton dust in preliminary exposures.

In one human study involving exposure to cotton dust, concentrations of several plant products and endotoxin were measured. FEV_1 responses to card-generated cotton dust from glanded cotton (containing gossypol) and to dust from glandless cotton (lacking gossypol) were equivalent at comparable airborne endotoxin concentrations but differing concentrations of dust, tannin, and terpenoid aldehydes (Rylander 1988). In an earlier study, acute FEV_1 responses to dust from cotton from which bracts had been removed prior to boll opening and to dust from cotton grown in an adjacent plot but with bracts left intact were equivalent at comparable airborne dust and endotoxin concentrations (Castellan et al. 1986). These observations support a causal role for endotoxin but not for bracts (Castellan et al. 1986) or the measured plant components (Rylander 1988).

Recent Epidemiological Studies in Cotton and Flax Mills

More than a dozen studies have been conducted and published on the possible association between byssinosis and inhalation of either endotoxin or GNB (the natural source of endotoxin in cotton and flax mills). With one exception discussed briefly above (Cavagna et al. 1969), all have been published since 1977. Table 68 summarizes these epidemiological studies.

Cinkotai's Surveys on Series of Symptoms

In a cross-sectional epidemiological survey, Cinkotai et al. (1977) evaluated prevalence rates of Monday chest symptoms among 720 workers employed in the cardrooms of several British cotton mills with very different dust concentrations and in a wool mill, a tea packing plant, and a tobacco factory. Participation rates were good, ranging from 81 to 100 percent of employees in each surveyed mill. Byssinosis prevalence in the various cotton mills surveyed ranged from 7 to 40 percent, while employees in the other mills lacked such symptoms. Overall analysis revealed no association between byssinosis prevalence and mean dust concentration (which ranged up to 15 mg/m^3 by plant), prompting the authors to comment that "the results clearly demonstrate the weakness of the [dust] standard designed to eradicate byssinosis."

In contrast, a very strong correlation ($r>0.95$, $p<0.001$) was observed between byssinosis prevalence and airborne-viable GNB concentration (which ranged up to 3,500 cfu/m^3 as quantified using Andersen impactor sampling and selective culture media). Endotoxin concentration, measured by a tube dilution method of the Limulus amebocyte lysate (LAL) clot test, was not highly correlated with byssinosis prevalence; however, the authors appear to have evaluated the correlation of symptom prevalence with endotoxin concentration in airborne dust (that is, per milligram) rather than with actual airborne endotoxin concentration (that is, per cubic meter). Cinkotai et al. (1977) concluded their report by stating that "the present investigation has demonstrated that airborne GNB in cotton mills are closely linked with the prevalence of byssinotic symptoms."

In a subsequent cross-sectional study, Cinkotai and Whitaker (1978) used the same survey methods in cardrooms of 21 major cotton spinning mills in Lancashire (including the 7 mills studied in their earlier investigation). Overall, 1,057 workers answered the questionnaire—a 93 percent participation rate. As in the earlier study, prevalence of Monday chest symptoms (which ranged from 0 to 40 percent by mill) was not significantly correlated with total dust (which ranged up to 3.85 mg/m^3 by mill) but was correlated ($r=0.66$, $p<0.002$) with airborne viable GNB (which ranged up to 6,150 cfu/m^3 by mill).

When interactions of the concentration and the number of years of exposure were evaluated, the strongest correlation also involved GNB ($r=0.73$, $p<0.001$). The correlation coefficient for GNB and Monday symptoms, however, was lower than in the previous study (Cinkotai et al. 1977), probably because the earlier study included noncotton facilities, which therefore had very low concentrations of GNB in the air and no occurrences of byssinosis. Endotoxin concentrations were not addressed. Cinkotai and Whitaker (1978) concluded that "the role of inhaled bacteria in the aetiology of byssinosis remains to be clarified."

Ten years later, Cinkotai again cross-sectionally surveyed the Lancashire textile industry (Cinkotai et al. 1988b; Cinkotai et al. 1988c, 1988d). Symptoms questionnaires were completed for 95 percent of the 4,903 workers employed in work areas ranging

Table 68. Results of epidemiological studies on cotton, flax, and hemp workers (limited to studies in which the effects of airborne endotoxin or airborne GNB were evaluated)

Study	Setting/ location	Maximal airborne concentrations* Dust (mg/m^3)	Endotoxin (ng/m^3)	GNB (cfu/m^3)	Number of workers studied	Results of study
Cavagna et al. (1969)	Cotton, textile, and hemp mills/ Milan	5.25 (TD)	8,710 (Soxhlet; TCA extract; rabbit Shwartzman)	Not tested	136	Monday symptoms correlated strongly with airborne endotoxin (r=+0.99, p<0.01) but not with total dust (p>0.12) in the four work areas studied.
Cinkotai et al. (1977)	Cotton, tobacco, tea, and wool mills/ Lancashire	15.00 (TDLF)	Not reported	3,500 (Andersen; endoagar with PCN)	678	Monday symptoms correlated highly with airborne GNB (r=+0.95, p<0.0001) but not with dust. Endotoxin concentration in dust as high as 1,600 ng/mg by LAL gel titration assay.
Cinkotai and Whitaker (1978)	Cotton and textile mills/ Lancashire	3.85 (TDLF)	Not tested	76,900 Andersen; endoagar with PCN)	1,057	Monday symptoms correlated with GNB (r=+0.66, p<0.002) but not with dust (r=+0.11).
Haglind et al. (1981)	Cotton and textile mills/ Sweden	2.00 (TDLF)	Not tested	112,000 (Andersen; Drigalski agar)	248	Monday symptoms correlated with GNB (r=+0.83, p<0.01) and also with dust (r=+0.62; p<0.05). Dust and endotoxin highly intercorrelated.
Diem et al. (1984)	Cotton and synthetic textile mills/ S.E. USA	1.88 (CAM)	1,110 (VE dust; H_2O extract; LAL gel titration)	110,093 (VE dust plated on selective TSA with CH and VM)	123	Monday FEV_1 change correlated weakly with log dust (r=+0.23, p<0.05) but not with log GNB or log endotoxin. However, population was very selected and spirometry and dust sampling were done a week apart. Dust and endotoxin were correlated.
Kawamoto et al. (1987)	Cotton, garnetting, and mattress plants/ California	>1.18 (VE)	100 (VE dust; H_20 extract; chromogenic LAL)	Not tested	128	No significant association of symptoms or of lung function with dust or endotoxin, but endotoxin exposures were very low (median = 5 ng/m^3).
Kennedy et al. (1987)	Cotton and textile mills/ Shanghai	2.50 (VE)	920 (VE dust; H_2O extract; chromogenic LAL)	Not tested	404	Stratified analysis showed a relationship between endotoxin (but not dust) and symptoms and baseline and across-shift FEV_1. Regression analysis showed that endotoxin (but not dust) was related to baseline FEV_1 and chronic symptoms (p<0.05).
Cinkotai et al. (1988b, c, and d)	Cotton and synthetic textile mills/ Lancashire	4.17 (TDLF)	580 (VE dust; H_2O extract; LAL gel	15,700 (Andersen; endoagar with PCN titration)	4,656	Many factors associated with Monday symptoms including endotoxin (p<0.001). Generally GNB levels and the prevalence of Monday symptoms were lower than in an earlier survey, but dust levels were not lower (Cinkotai 1978).

continued on next page

Table 68. Results of epidemiological studies on cotton, flax, and hemp workers (limited to studies in which the effects of airborne endotoxin or airborne GNB were evaluated)—*Continued*

Study	Setting/ location	Maximal airborne concentrations* Dust (mg/m³)	Endotoxin (ng/m³)	GNB (cfu/m³)	Number of workers studied	Results of study
Cinkotai et al. (1988a)	Flax mills/ Normandy	47.10 (TDLF)	Not tested	67,900 (Andersen; endoagar with PCN)	308	Dust and GNB related to chronic bronchitis, but with Monday symptoms. Very high dust and GNB levels and unexpectedly low occurrence of Monday symptoms suggest likely self-selection of workers.
Niven et al. (1991)	Cotton and synthetic textile mills/ Manchester	Not reported (TDLF; TD; personal TDLF)	Not reported (turbidometric LAL)	Not tested	1,893	Monday symptoms most strongly associated with log endotoxin by personal sampling (p<0.001).

**CAM*, Continuous aerosol monitor (ppm, Inc., Knoxville, TN). *CH*, Cycloheximide. *PCN*, Penicillin. *TCA*, Trichloroacetic acid. *TD*, Total dust (area). *TDLF*, Total dust, less fly (area). *TSA*, Trypticase soy agar. *VM*, Vancomycin.

from opening areas to weaving areas at the 31 mills surveyed (including four mills that exclusively processed synthetic fibers). Average work area samples of total dust (ranging up to 4.02 mg/m³) were collected as before. Concentrations of GNB ranging up to 1,150 cfu/m³ were measured. A portion of these dust samples were assayed for endotoxin (average concentration ranging up to 580 ng/m³ by work area). Also, personal total dust samples and personal respirable dust samples were collected in workers' breathing zones. In addition to airborne endotoxin concentration (p<0.001), numerous other interrelated factors were associated with the prevalence of chest symptoms on Mondays.

Cinkotai et al. (1988c) commented that "the effects of airborne endotoxins...could not be fully evaluated because the available data set was not complete." Although a statistical model of the data developed by stepwise logistic multiple regression did not include endotoxin concentration as a prediction factor, it did include two other factors that are likely to have been related to endotoxin concentration, namely work area (Castellan et al. 1988) and cotton quality (Olenchock et al. 1983). The reduced prevalence of Monday chest symptoms relative to 10 yr earlier and the airborne concentrations of GNB above 300 cfu/m³ in only three work areas (all cardrooms with among the highest prevalences of byssinotic symptoms) led Cinkotai et al. (1988c) to suggest that "the most important single factor causing the decline in the prevalence of byssinotic symptoms (PBS) may be the disappearance of bacteria from the workroom air. The PBS values peaked in the very three factories where airborne bacteria were observed in significant concentrations . . . . However, since last surveyed about 10 yrs ago . . . their overall decline was quite conspicuous and was not paralleled by a similar decline in airborne dust levels."

This comment was echoed in another report (Cinkotai et al. 1988b) by the statement that "the downward trend in the prevalence of byssinotic symptoms may be associated with the disappearance of much of the bacteria from workroom air." As there had been no specific program to decrease worker exposure to bacteria, the authors suggested that "the quality of dust, however, may have changed, due to the use of cleaner raw cotton and the closure of plants that processed the particularly dirty, coarse fibre." (Cinkotai et al. 1988b)

More recently, Cinkotai et al. (1988a) cross-sectionally surveyed 12 flax mills in Normandy, France, an area noted by the authors as generally having a low prevalence of byssinosis according to previous unpublished reports. Airborne endotoxin was not measured in this study, but mean area total dust

concentrations (up to 47.1 mg/m^3) and mean viable GNB concentrations (up to 67,900 cfu/m^3) were both exceptionally high. Over 90 percent of the 340 people employed in these mills answered a symptoms questionnaire. Overall, the byssinosis prevalence rate was only 12.5 percent, an unexpectedly low figure considering the high levels of dust and GNB exposures. Using a chi-square analysis instead of a correlation analysis (as was done in the previous reports), the authors reported a lack of statistically significant associations between Monday chest symptoms and either airborne dust or GNB concentrations. In addition to difficulty translating the questionnaire, the authors suggested other possible explanations for the unexpectedly low prevalence of Monday symptoms, including self-selection of susceptible workers out of the workplaces with extremely high levels of exposure. Considering these caveats, Cinkotai et al. (1988a) concluded that "it cannot rightly be inferred from the present observations that flax dust or bacteria are not in some way associated with byssinosis."

Bale Bacterial Count and Decreases in Across-Shift FEV_1

Rylander et al. (1979) used results of a company-operated medical monitoring program involving nearly 4,000 cardroom workers in 23 U.S. cotton mills to investigate changes in FEV_1 over a Monday workshift. Three indices of exposure—mean cardroom airborne dust concentration as measured by vertical elutriator (ranging up to 1.35 mg/m^3), mean concentration of GNB cultured from cotton fiber sampled from bales processed in the mills studied, and cotton/synthetic blend ratio—were used as independent variables in various linear and nonlinear statistical models describing the relationship between exposure and across-shift change in FEV_1. A mean across-shift decrement in FEV_1 was determined for each mill, and each mean value was weighted by the number of examined employees at that mill. Although a statistically significant linear relationship between log dust concentration and decline in FEV_1 was found (r=0.50, p<0.01), a stronger correlation (r=0.62, p<0.01) was observed when FEV_1 was correlated with the square root of the product of viable GNB (sampled from bales) times airborne dust concentration adjusted for blend ratio. (In a preliminary report from the first 15 mills studied, Rylander and Lundholm (1978) reported that when across-shift Monday FEV_1 decline was correlated separately with exposure to airborne dust and GNB, the simple correlation coefficients were 0.46 and 0.78, respectively.)

The medical monitoring program from which the data were obtained had been in operation for several years in the studied mills, and Rylander et al. (1979) explained that workers previously found to be affected by dust had been transferred to jobs involving lower dust exposures. In addition, spirometry testing, dust measurements, and bale sampling did not temporally coincide. All these factors were likely to have limited the authors' ability to observe stronger associations between GNB and acute FEV_1 decline even if they had existed. Most limiting, in terms of the overall importance of this study, is the fact that neither airborne GNB nor airborne endotoxin concentrations were measured.

Swedish Symptoms Survey

In a survey of the Swedish cotton textile industry, Haglind et al. (1981) interviewed 86 percent of workers in the opening, carding, and spinning areas. The prevalence of Monday chest symptoms by work area correlated with the concentration of total airborne dust (which ranged up to 2.0 mg/m^3) and the concentration of airborne-viable GNB (which ranged up to 200 million cfu/m^3).

Analyzing all surveyed work sites, Haglind et al. (1981) observed a stronger correlation of Monday chest symptoms with airborne GNB (r=0.73, p<0.01) than with airborne dust (r=0.62, p<0.05). Excluding one work site that processed medical grade cotton, the correlation with dust improved (r=0.81, p<0.001), but the GNB correlation was still reasonably high and statistically significant (r=0.71, p<0.01). Data published in their report indicate that the two measured environmental indices were highly correlated (r=0.68, p<0.008 for all sites; and r=0.90, p<0.001 excluding the medical cotton site), which could explain why both correlated with byssinosis prevalence. Given this correlation between endotoxin and airborne dust, the results of this study cannot be accepted as strong evidence for an endotoxin effect in the absence of a dust effect.

A later study conducted at one of these Swedish mills by Rylander et al. (1983), although an observational study of worker responses to their workplace exposures, must be categorized as clinical rather than epidemiological. Data were collected from only 15 workers (2 of whom were excluded from the analysis), and Rylander et al. (1983) commented that "as the technical capacity of the study was limited, no further attempts were made to recruit additional workers." Thus, as indicated by the authors, little import can be given to the lack of a significant relationship between personal exposures to dust or endotoxin and individual across-shift change in FEV_1. Means of data grouped by work area were more consistent with an endotoxin effect than a dust effect, but low numbers of observations limited analysis of these data.

Endotoxin and Acute FEV_1 Declines in U.S. Textile Mills

Diem et al. (1984) reported a preliminary analysis of some data from the first year of a 5-yr longitudinal respiratory health study of workers employed at selected U.S. cotton textile mills. In a multiple regression analysis of data from 123 male nonsmokers and smokers, acute change in lung function over a Monday workshift was observed to be significantly (though not strongly) correlated with the logarithm of airborne, vertically elutriated dust concentration (r=0.23, p<0.05), but not with either the logarithm of airborne GNB concentration (r=–0.01) or with the logarithm of airborne endotoxin concentration (r=–0.09).

Potential limitations of this study include a highly selected population (having survived a well-established medical monitoring program intended to remove the most susceptible workers), a very strong correlation among the environmental indices (r=0.78 to 0.92), a temporal difference (1 wk) between pulmonary function testing and environmental measurements, and relative imprecision of the titration gel method used as compared with other endotoxin assays (for example, the chromogenic modification of the LAL assay). Thus, the results cannot be accepted as substantial evidence against an endotoxin effect in the absence of a dust effect.

Airborne Endotoxin in a Cotton-Garnetting Mill

Kawamoto et al. (1987) reported on a respiratory health and environmental survey of workers who manufacture padding and mattresses in the 13 cotton-garnetting facilities located in California. The cotton used in these facilities was waste cotton from gins and cottonseed oil mills located in California. Of 193 English- or Spanish-speaking dayshift workers employed, only 66 percent participated in at least some part of the survey. Complete exposure data and before-and-after-shift pulmonary function data were available for only 30 percent of the workers.

Overall, 10 percent of the workers had Monday chest symptoms. Although Kawamoto and associates failed to observe statistically significant associations between acute or chronic chest symptoms or acute or chronic pulmonary function changes and either dust or endotoxin concentration, a trend toward larger decreases in Monday FEV_1 with higher endotoxin exposures is evident in their tabulated data. This trend was not found to be statistically significant (no p value was reported), but the authors used an overly conservative Scheffe method for multiple comparisons (Boardman and Moffitt 1971). Moreover, although the authors claimed that their analysis had adequate statistical power, they seemed to believe that a mean workshift-related change in FEV_1 must be 5.3 or greater before it is worth detecting. In this regard, it is noteworthy that results of the three earlier garnetting and waste cotton studies reviewed by Kawamoto et al. indicate that a mean FEV_1 change this large was unlikely to have been observed.

Bias to the null in the Kawamoto et al. (1987) study may also have resulted from selection factors resulting in the low worker participation rate. Beyond that, only stratified analyses of grouped data were performed, which may have resulted in a potential loss of information from continuous variables (for example, individual measures of endotoxin concentration, dust concentration, and workshift-related change in FEV_1).

The most obvious limitation of this study in terms of its inability to turn up an observable relationship between measures of respiratory morbidity and

endotoxin exposure was that endotoxin exposures were generally very minimal (median level of 5.2 ng/m^3). Very low endotoxin exposures in these California garnetting facilities is consistent with published reports of relatively low GNB and low endotoxin contamination of cotton grown in California (Simpson and Marsh 1985, Castellan et al. 1988). Largely on the basis of this last point, Kawamoto and colleagues appropriately concluded that their negative findings were "not inconsistent with studies that have found dose-response relationships between endotoxin exposure and decreases in FEV_1 during the workshift."

Endotoxin and Respiratory Effects in Shanghai Mills

Kennedy et al. (1987) reported a survey of Chinese cotton mill workers who were more highly exposed (vertically elutriated dust concentrations of up to 2.50 mg/m^3 and vertically elutriated endotoxin concentrations of up to 920 ng/m^3). Acute byssinotic effects could not be assessed in terms of typical Monday chest symptom patterns because of unusual work schedules. A 90 percent participation rate was achieved, but pulmonary function data from an additional 10 percent were excluded from analysis on the basis of nonreproducibility of test results as defined by guidelines of the American Thoracic Society (American Thoracic Society 1979). Use of the 1979 reproducibility criteria are now known to effect substantial study bias by differentially excluding from analysis the more functionally impaired study subjects (American Thoracic Society 1987).

In addition Kennedy et al. (1987) presented evidence that the most heavily exposed group of workers had been influenced by other selection pressures and that remaining workers in this group represented a less affected survivor population. When the highest exposure group was excluded from the analysis, a stratified analysis revealed clear and consistent trends for an endotoxin effect and the absence of a dust effect (on both baseline and decreased across-shift FEV_1, and on both acute and chronic chest symptoms).

In a regression analysis, although the coefficient for airborne endotoxin concentration was not significantly related to a decrease in across-shift FEV_1, it was significantly related to baseline FEV_1 ($p < 0.01$), and both coefficients were substantial. Even in a subgroup of cotton workers who had relatively low endotoxin exposures, Kennedy et al. (1987) observed excesses of acute and chronic chest symptoms and larger decreases in across-shift FEV_1 ($p<0.06$) compared with silk workers. This observation suggested to Kennedy et al. (1987) that "even exposure to endotoxin at 1 to 20 nanograms per cubic meter constitutes an 'adverse respiratory health effect' . . . ." However, because of the cross-sectional nature of the study and because no environmental sampling was done at the silk mill used for comparison, this particular conclusion must be viewed with caution.

A Recent Study of British Textile Workers

A recent preliminary report (Niven et al. 1991) summarized the findings of a survey of 1,093 British textile workers and an environmental survey of the seven cotton and two synthetic fiber mills in which they were employed. Exposures to dust were measured by sampling work areas and personal breathing zones. Exposures to endotoxin were measured using a turbidometric modification of the LAL assay applied to personal dust samples. Byssinosis symptom prevalence was most strongly correlated with log endotoxin ($p<0.001$), suggesting that endotoxin might be an etiological factor.

Beyond Byssinosis

Recent reports from atypical settings add considerable support to the contention that endotoxin inhalation causes byssinosis. For example, 22 percent of the 303 daytime workers at a wool carpet-weaving factory reported Monday symptoms typical of byssinosis (Ozesmi et al. 1987). They were exposed to airborne dust (up to 4.4 mg/m^3) with high endotoxin contamination (up to 31.2 μg/g). Moreover, as in byssinosis, symptomatic individuals were shown to have a decreased across-shift FEV_1 especially on Monday as compared to other weekdays. The entirely different nature of this dust compared to cotton textile mill dust suggested to Ozesmi et al. (1987) that "the finding of endotoxin together with the absence of cotton confirms the theory that

'byssinosis' is due to bacterial endotoxin rather than to cotton per se."

A Monday pattern of symptoms is becoming increasingly observed in cases of "humidifier lung," a condition with many clinical similarities to byssinosis (Ganier et al. 1980). In one reported outbreak at a printing facility with a microbiologically contaminated humidifier, 40 percent of 50 employees experienced fever and 24 percent experienced chest tightness temporally associated with humidifier operation (Rylander and Haglind 1984). Airborne endotoxin concentration after 2 hr of humidifier operation was measured to be as high as 390 ng/m^3.

In another work environment in which acute and chronic airway symptoms were prevalent in employees, a clear relationship between exposure to airborne endotoxin and a decrease in across-shift FEV_1 was observed in data from 57 workers in swine confinement buildings (Donham et al. 1988). Others have also observed a relationship between endotoxin exposure and airflow impairment in swine confinement workers (Zejda et al. 1991).

In animal feed mills, Heederik et al. (1991) have observed a strong negative association of dust and endotoxin with preshift ventilatory function, but "cumulative endotoxin was more strongly related to decreases in lung function than the dust exposure." This suggests that endotoxin exposure was an important factor in the development of chronic impairment of airway function in these workers.

The exposure to airborne endotoxin and associated respiratory syndromes, including decreases in FEV_1, have been documented but not well analyzed among other occupational groups, including poultry farmers (Thelin et al. 1984), silo unloaders (Pratt and May 1984), and grain workers (doPico et al. 1986). Ongoing and future research in these and other areas is likely to shed additional and substantial light on endotoxin as an etiologic agent of occupational respiratory disease.

Published Suggested Limits for Airborne Endotoxin Exposure

As reviewed in other chapters of this book, the existing evidence on the effects of endotoxin has driven a substantial ongoing research effort to identify the factors that influence the contamination of cotton with endotoxin and to pilot intervention strategies to substantially reduce this contamination. In addition, as summarized briefly in the following paragraphs, the existing evidence has resulted in the publication of several suggested levels at which to limit occupational exposure to airborne endotoxin.

Rylander and Lundholm (1978), while recognizing that a definite causal role in the etiology of byssinosis had not yet been established, suggested that "it may be prudent to establish a standard for airborne GNB, to supplement the existing dust standard." A decade later, after endotoxin testing had become more standardized and routine, Rylander (1987) proposed that a standard for endotoxin should be developed for specific application to cotton dust. Rylander (1987) listed endotoxin-based "tentative thresholds for symptoms after exposure to cotton dust," which included "fairly certain" thresholds of 0.5–1.0 μg/m^3 for fever, 0.3–0.5 μg/m^3 for chest tightness, and 0.1–0.2 μg/m^3 for decreases in across-shift FEV_1, [even though Rylander et al. (1985) had previously published a report indicating a lower threshold—33 ng/m^3—for decreases in across-shift FEV_1]. In accord with the interpretation by Kennedy et al. (1987) that chronic respiratory effects may occur at airborne endotoxin exposures in the range of 1–20 ng/m^3, Rylander (1987) listed an "uncertain" threshold of 0.02 μg/m^3 for chronic bronchitis.

Popendorf (1986) proposed a general guideline of 0.1 μg/m^3 as a potentially hazardous airborne concentration for occupational settings. Several years later, while indicating the need for further research before a mandatory Federal standard could be established for airborne endotoxin, Jacobs (1989) called for the establishment of an endotoxin "threshold limit value" to guide voluntary control of this occupational health hazard. In fact, a genetic engineering company has used data from cotton dust exposure studies to establish its own voluntary airborne endotoxin action level at 30 ng/m^3 (Palchak et al. 1988). Concentrations above this level trigger use of personal protective devices, intensified environmental monitoring, medical surveillance, and appropriate additional engineering and administrative controls to protect worker health.

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